Genetic and physical interaction of Sgt2 protein with prion-chaperone machinery

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Genetic and physical interaction of Sgt2 protein with prion-chaperone machinery

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Summary

The word “Prion” refers to self-perpetuating protein aggregates that cause neurodegenerative diseases in mammals. It is a protein isoform that has undergone a conformational change which converts the normal form of the protein into the infectious form with the same amino acid sequence.

Yeast [*PSI*$^{+}$] prion is the prion isoform of Sup35 protein, a translation termination factor eRF3. It has been suggested that prion [*PSI*$^{+}$] is controlled by the ensemble of chaperones with Hsp104 playing the major role. The previous work performed in the Chernoff’s lab showed that the defective GET pathway caused by *get2*Δ led to the defect in [*PSI*$^{+}$] curing by excess Hsp104. The GET pathway is a system responsible for transporting newly synthesized TA-protein to the ER membrane, and the components which have been proven to be involved in this pathway include: Get1, Get2, Get3, Get4, Get5 and Sgt2.

In this study we describe the mechanism underlying the effect of the defective GET pathway on [*PSI*$^{+}$]. We demonstrate that Sgt2, one of the components of GET pathway, interacts with Sup35 in both [*PSI*$^{+}$] and [*psi*$^{-}$] strains through its prion domain. Overproduction of Sgt2 and Hsp70-Ssa is triggered by the defective GET pathway and

leads to the protection of [*PSI*$^{+}$] aggregates from curing by excess Hsp104. We show that the direct interaction between Sgt2 and Hsp70-Ssa is not required for this protective effect.

1 Introduction

1.1 Prions

In molecular biology, it was well known that the information is transmitted only by nucleic acids like DNA and RNA. However, the discovery of the protein-based inheritance led to a change in the central dogma of molecular biology. The name for these heritable protein determinants - prion- is a portmanteau derived from the combination of two words *protein* and *infection*, since they have been proven to be an infectious agent in mammals[1]. We now know that the inheritable information can be efficiently transferred from cell to cell though cytosol by prion proteins. Neither the original nucleotide sequence of the gene or mRNA nor the derivative amino acid sequence of the prion protein is changed compared to the normal cellular form of the same protein. Nevertheless, the prion is efficiently transmitted to the progeny in a non-Mendelian pattern after segregation in meiosis [2].

The term prion was originally introduced over 30 years ago by S. Prusiner to explain the unusual transmission pattern of neurodegenerative diseases[1]. Prion is a protein isoform that has undergone a conformational change which converts the normal form of the protein into the infectious form with the same amino acid sequence. This change

diminishes its own ability to perform the normal function but the infectious ability enables it to propagate [3,4]. (see Figure 1).

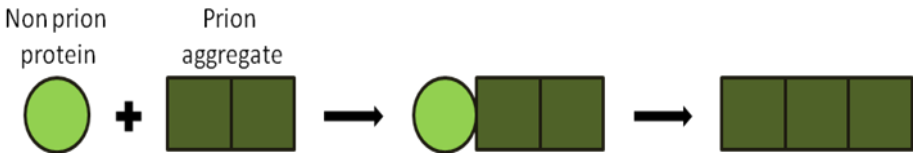


Figure 1 Prion model
Aggregated prion form of the protein (square) converts a non-prion form (circle) of the same protein.

Prion propagates by transmitting a misfolded protein state in an infectious way. When prion enters an organism, it induces existing, properly-folded proteins to convert into the prion form. Prion acts as a template to guide the alternative folding of the native protein into prion conformation. These newly-formed prions can then go on to convert more proteins and a chain reaction is triggered that produces large amounts of the prion form. All known prions induce the formation of amyloids, the extremely stable fibrils of tightly packed beta sheets [5,6].

1.2 Prion diseases

A big part of all known mammalian prion diseases, collectively called transmissible spongiform encephalopathies (TSEs)[7], such as “mad cow disease”, or bovine spongiform encephalopathy (BSE), scrapie disease of sheep, and human Creutzfeldt-Jacob disease, are

untreatable and fatal and characterized by spongiform degeneration of the brain. Other human manifestations include Gerstmann-Straussler-Scheinker disease (GSS), Kuru, and Fatal familial insomnia [8-10].

These prion diseases are caused by the so-called prion protein, PrP. The amyloid structure of prion is extremely stable and accumulates in infected tissue, and prion diseases are caused by prion protein aggregating extracellularly within the central nervous system and forming amyloid plaques, which disrupt the normal tissue structure. This disruption is characterized by "holes" in the tissue with resultant spongy architecture due to the vacuole formation in the neurons[6,9,11]. Also, it has been proposed that neurodegeneration caused by prions may be related to the lost of normal function of PrP[12]. Evidence showed that losing PrP may have an abnormal function in maintenance of long-term memory[13,14]. Moreover, studies showed that PrP expression on stem cells is necessary for an organism's self-renewal of bone marrow[15]. However, the real physiological function of the prion protein remains a controversial matter.

The properties of PrP are very similar to those seen in various non-infectious amyloids resulting from conversion of certain proteins or their fragments from the normally soluble form to the insoluble fibrils or plaques, which place prion diseases into the large and

heterogeneous group of amyloid diseases, including about 20 human diseases, such as Alzheimer's disease, Huntington's disease and Parkinson's diseases[16-18]. These diseases occur widely in humans and other mammals, and the mechanism by which such conformational switches lead to aggregate formation and disease remains unknown. The auto-catalyzed misfolding, perpetuation of wrong conformation and aggregation of proteins in these amyloid diseases are consistent with the prion concept observed in TSEs, which make studies related to prion not only benefit the understanding of TSEs but also amyloid diseases.

1.3 Yeast prions

Investigating prion in mammals is a tough task since the key experiments are often infeasible. Fortunately, there is a simple eukaryotic model for researching prion biology - the budding yeast *Saccharomyces cerevisiae*. Yeast were shown to have multiple factors with typical prion behavior, which makes them a very simple and convenient model for studying the mammalian prions[19]. Yeast cells are easily manipulated and cultured in the laboratory, and those powerful standard techniques developed in yeast, such as yeast two-hybrid, synthetic genetic array analysis and tetrad analysis, could be used to understand the mechanism of prion propagation. Although prions in yeast are sometimes associated with certain toxicity phenomena,

however, by themselves, yeast prions are not pathogenic.

A set of genetic criteria was proposed for researchers to identify prions in yeast. The criteria include: non-Mendelian inheritance, reversible curability and inducibility by overexpressing the normal protein isoform. Three of the best-characterized prions of *S. cerevisiae* are [*URE3*], [PIN^+] and [PSI^+], and all of them meet the criteria for identifying prions in yeast (see Table 1). They are inherited in a non-Mendelian manner and require specific cellular proteins for maintenance (Ura2 gene in case of [*URE3*], Sup35 gene in case of [PSI^+], and Rnq1 in case of [PIN^+]). They are reversibly curable using agents such as guanidine hydrochloride, and the prion form can be induced by the over expression of the related protein.

Table 1 Known yeast prions

Prion	Protein	Normal Function
[URE3]	Ure2	Nitrogen catabolism repressor
[PIN$^+$]	Rnq1	Unknown
[PSI$^+$]	Sup35	Translation termination factor
[MCA$^+$]	Mca1	Putative yeast caspase
[Swi$^+$]	Swi1	Chromatin remodeling
[OCT$^+$]	Cyc8	Transcriptional repressor
[MOT3$^+$]	Mot3	Nuclear transcription factor

[*URE3*] is the prion form of the protein Ure2 that is a nitrogen catabolic repressor. Because of the partial loss of function of Ure2 in the presence of [*URE3*], cells are able to uptake poor nitrogen sources of uracil, which gives a prion the phenotype of growth on media lacking uracil[20]. [*PIN*$^+$] is the prion form of the protein Rnq1 with unknown normal cellular function. The presence of the prion [*PIN*$^+$] facilitates the *de novo* formation of the prion [*PSI*$^+$] [21]. [*PSI*$^+$] is the prion forms of the protein Sup35 that functions as a translation termination factor eRF3. The presence of [*PSI*$^+$] is detected by the partial loss of function of Sup35 [22].

1.4 Yeast prion [*PSI*$^+$]

In this study, we focused on [*PSI*$^+$] - prion form of Sup35. The protein Sup35 could be divided into three structurally and functionally distinctive domains: the N domain, the M domain and the C domain (see Figure 2). The N-proximal (Sup35N) domain is required for [*PSI*$^+$] induction and propagation. The middle M domain (Sup35M) is involved in the control of stability of prion [*PSI*$^+$]. The C-terminal domain (Sup35C) is essential for translation termination and viability [22] [23] [24]. In the prion aggregates the sequence of N domain has been proven to be arranged in a double layer of β-sheets (see Figure 3 and Chapter1.1), typical for other amyloids, and each sheet is held together by hydrogen bonds between side chains and between the backbones[25].

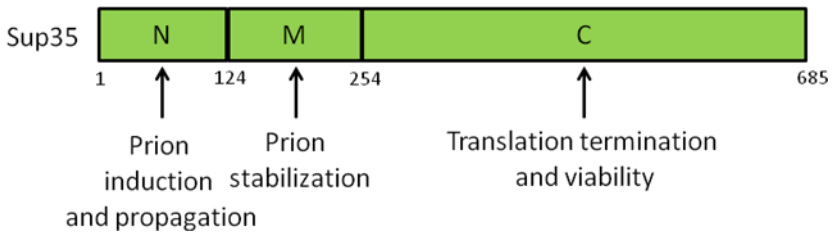


Figure 2 Structural and functional organization of the Sup35 protein
N, M and C refer to Sup35N, Sup35M and Sup35C domains, respectively

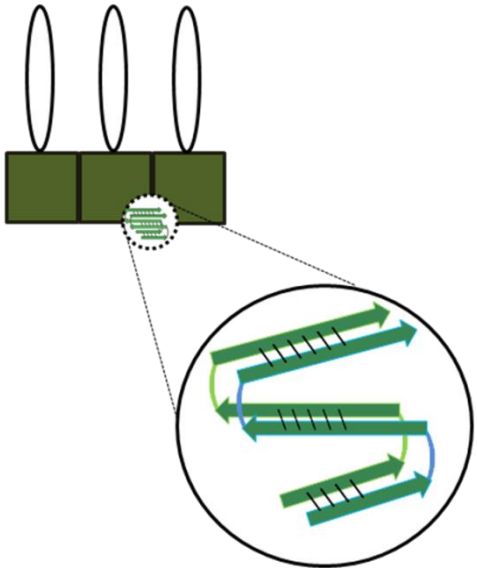


Figure 3 Structural organization of [PSI$^+$]

C-terminal domains of Sup35 (white circles) are exposed on the side. Prion domains (green squares) form an axis. Inset depicts a detailed structural view of the prion domain, that the strands of prion domain (green arrows) are parallel and precisely aligned to form the β-sheet.

Sup35 normally works as the eukaryotic release factor eRF3. In the process of translational termination, eRF1 protein recognizes the stop codon and catalyzes the hydrolysis of the bond between the nascent polypeptide and tRNA, in which eRF3 works as a GTPase that binds to eRF1 to facilitate the release activity. In yeast, mutations in the *SUP35* gene result in the suppression of all three types of stop codons (*UAA*, *UAG*, and *UGA*), a phenomenon known as nonsense suppression[26 27].

In the prion form most of Sup35 is aggregated, that causes the loss of its normal activity and nonsense-suppression in [*PSI*$^+$] cells. The most convenient phenotypic

assay for detecting [*PSI*$^{+}$] is based on this effect. *S. cerevisiae* strains used in this study carry *ade1-14*$_{UGA}$ nonsense-mutation. In the mutant strain the Ade1 protein involved in the adenine biosynthesis is truncated and is not functional due to the premature translation termination. In [*PSI*$^{+}$] cells, most of Sup35 is aggregated, that leads to the readthrough of the premature stop codon and synthesis of full-length Ade1 protein from *ade1-14* transcript. The production of full-length Ade1 allows the strains to grow on media lacking adenine, the phenotype for [*PSI*$^{+}$] detection in this study. In [*psi*$^{-}$] cells, the absence of functional Ade1 protein prevents growth on media lacking adenine [28] [29] (see Figure 4).

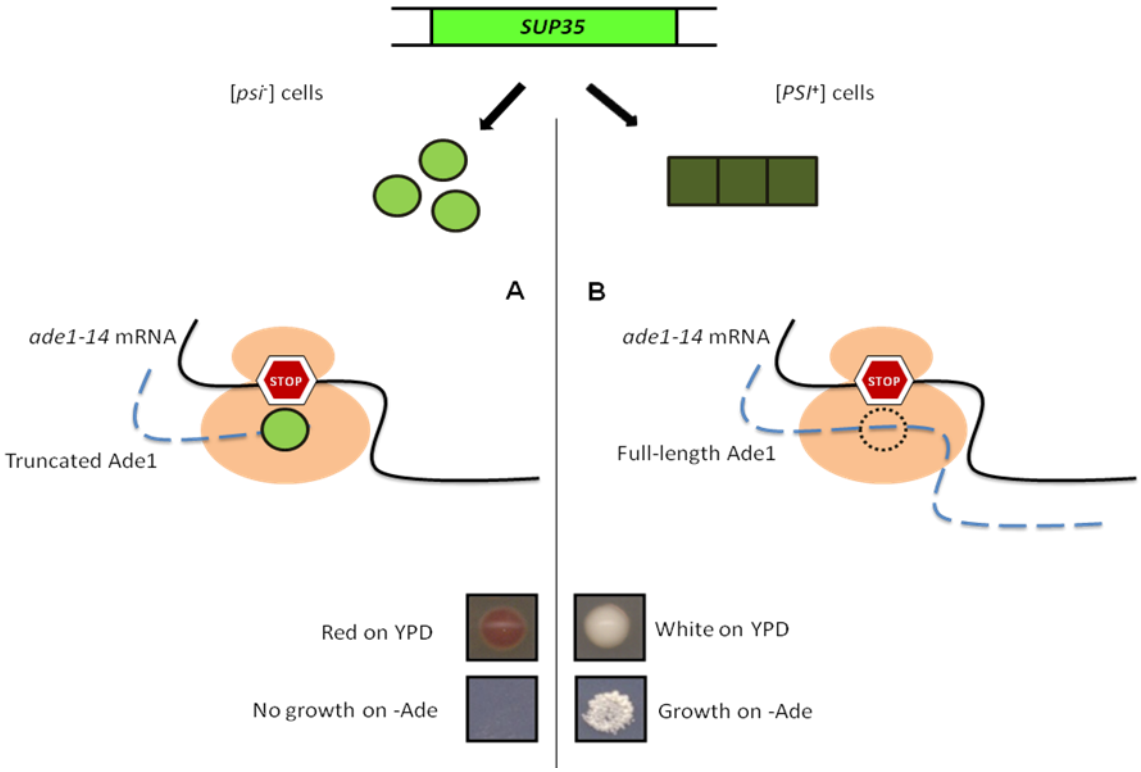


Figure 4 Experimental assay to detect [PSI$^+$]

A) Sup35 in the non-prion form efficiently participates in recognition of stop codons. A nonsense mutation in *ade1-14* mRNA leads to the premature translation termination and synthesis of the truncated Ade1 protein. The lack of full-length Ade1 causes poor growth on –Ade medium and a red color of the strain on YPD. B) In [*PSI*$^+$] cells, most of Sup35 is aggregated, that leads to the readthrough of the premature stop codon and synthesis of full-length Ade1 protein from *ade1-14* transcript. The production of full-length Ade1 causes growth on –Ade medium and a white color on YPD.

1.5 [*PSI*$^+$] and chaperon Hsp104

Chaperones, also called heat shock proteins (Hsps), is a group of proteins that facilitate the proper folding of nascent proteins, prevent the aggregation of denatured and damaged proteins, refold misfolded proteins, and assist the degradation of proteins that cannot be properly refolded. Chaperones are induced when cells undergo various types of stresses like heat, cold, oxygen deprivation. Among different families of chaperones

the three groups are the most important for prion propagation in yeast: Hsp104, Hsp70s, and Hsp40s [30].

The first chaperone protein found to have an effect on prion was Hsp104, and it was proven to be essential for the propagation of [PSI^+][31]. Hsp104, a homologue of prokaryotic ClpB, is an yeast member of the evolutionarily conserved Hsp100/ClpB family and acts as an ATPase in the form of homohexamer. The normal cellular function of Hsp104 is disaggregation of stress-damaged proteins and it is the only chaperone known so far to be able to act on aggregated proteins to affect their disaggregation, while other chaperones are believed to act by preventing the aggregation[32]. There is no homologue of Hsp104 found in mammals. However, with the importance of its ability to solubilize aggregated proteins, Hsp104 is believed to have a functional homologue in mammalian systems. These characteristics make the study of the role of Hsp104 in [PSI^+] propagation and disaggregation so important for the understanding of the mechanisms of amyloid diseases.

To explain the unique relationship between Hsp104 and [PSI^+] the model based on the Hsp104 levels was suggested [31,33,34](see Figure 5). Moderate levels of Hsp104 are required to break the [PSI^+] aggregates into smaller seeds which initiate new rounds of

propagation. Both the depletion and overproduction of Hsp104 cure [*PSI*$^{+}$]. Depletion of Hsp104 leads to the increase of prion aggregates in size but decrease number of aggregates, which reduces the efficiency of prion transmission into daughter cells and its loss in cell culture. The Hsp104 overproduction leads to the elimination of [*PSI*$^{+}$] (the [*PSI*$^{+}$] "curing"). Potentially, the overproduction provides sufficient resources to break the aggregates into monomers that are refolded by chaperone system or degraded by ubiquitin-proteasome pathway. Other chaperones also have their own effects on [*PSI*$^{+}$] curing by excess Hsp104. For examples, two sub-categories of Hsp70s family, Ssa and Ssb, had been shown to affect the curing when overproduced. While excess Ssb increases the efficiency of this [*PSI*$^{+}$] curing the curing is antagonized by excess Ssa[35]. The complex nature of interaction of multiple chaperones with [*PSI*$^{+}$] suggests the presence of the mechanisms regulating these interactions.

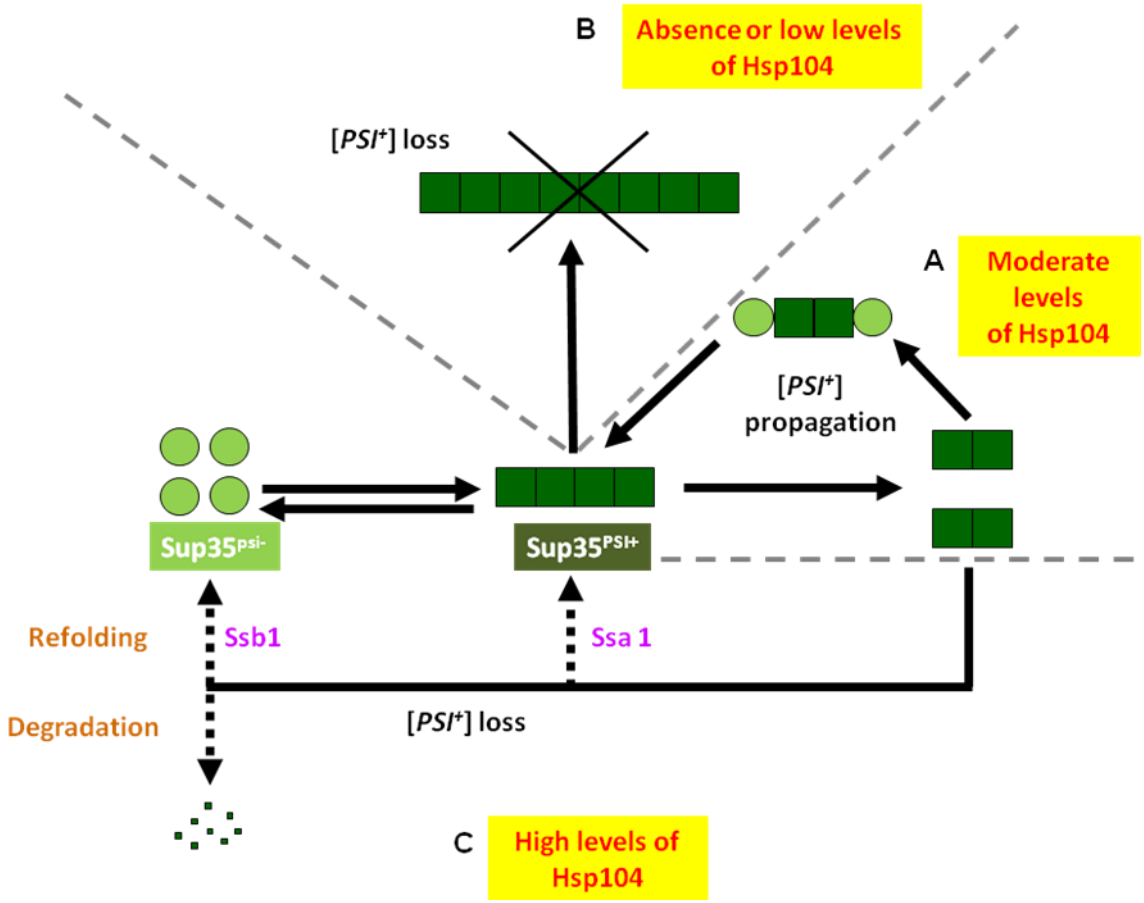


Figure 5 Roles of Hsp104 in [PSI$^+$] propagation and degradation

A) Moderate levels of Hsp104 are needed for efficient formation of the new prion "seeds". B) Inhibition or loss of Hsp104 function lead to the increase of prion aggregates in size but decrease number of aggregates, that reduces the efficiency of prion transmission into daughter cells. C) Hsp104 overproduction leads to the elimination of [*PSI*$^+$] (the [*PSI*$^+$] "curing"). Potentially, the aggregates are broken into monomers that are degraded by ubiquitin-proteasome pathway or refolded by chaperone system.

1.6 GET system

A project was carried out in the Chernoff's lab to find factors that regulate the curing of [*PSI*$^+$] by excess Hsp104. Using EMS mutagenesis 13 colonies with the defect of [*PSI*$^+$] curing by Hsp104 overexpression were isolated. One of these clones with the

stronger defect of curing was found to have a premature stop codon in the nucleotide position 473 of the *GET2* gene.

Get2 is a component of the Guided Entry of Tail-anchored proteins (GET) pathway (see Figure 6) which is required for the targeted delivery of the Tail-anchored (TA) proteins from the ribosome to the endoplasmic reticulum (ER)[36]. Other known components of the GET pathway are Get1, Get3, Get4, Get5, and Sgt2. Newly synthesized TA protein is recognized by the C domain of Sgt2-Get5-Get4 complex. In this complex, Get5 interacts with the N domain of Sgt2 and Get4 interacts with Get5. Then the TA protein-Sgt2-Get5-Get4 complex is formed, in which protein components are centered around Sgt2. The TA protein is then handed off to Get3 which is stabilized in its closed conformation by Get5/Get4 for facilitating Get3-TA protein complex formation. The Get3-TA protein complex is recruited to the ER membrane by interaction with Get1/Get2 complex anchored in the ER membrane[37]. After ATP hydrolysis, conformational change of Get3 brings it back to the opened conformation and releases the TA protein which is inserted in the ER membrane. Get3 is then recycled[38]. In the absence of Get2 Get1/Get2 complex is not functional and TA-proteins fail to reach the ER membrane and form cytosolic aggregates. The cytosolic components of GET pathway (Sgt2, Get3, Get4, and Get5) are sequestered by

the TA aggregates[36]. Sgt2 is a small glutamine-rich cytoplasmic protein described as a potential cochaperone since its vertebrate homologue, SGT, has been known as a cochaperone that regulates Hsp70s. Sgt2 contains tetratricopeptide repeats (TPR), which often mediate protein-protein interactions. Structurally and functionally Sgt2 could be divided into three domains: the N-terminal domain, the middle TPR domain and the C-terminal domain (see Figure 7). The N domain is responsible for interaction with Get5.The TPR domain has sites of interaction with chaperones Hsp104/70/40s. The C domain is essential for TA protein recognition[37].

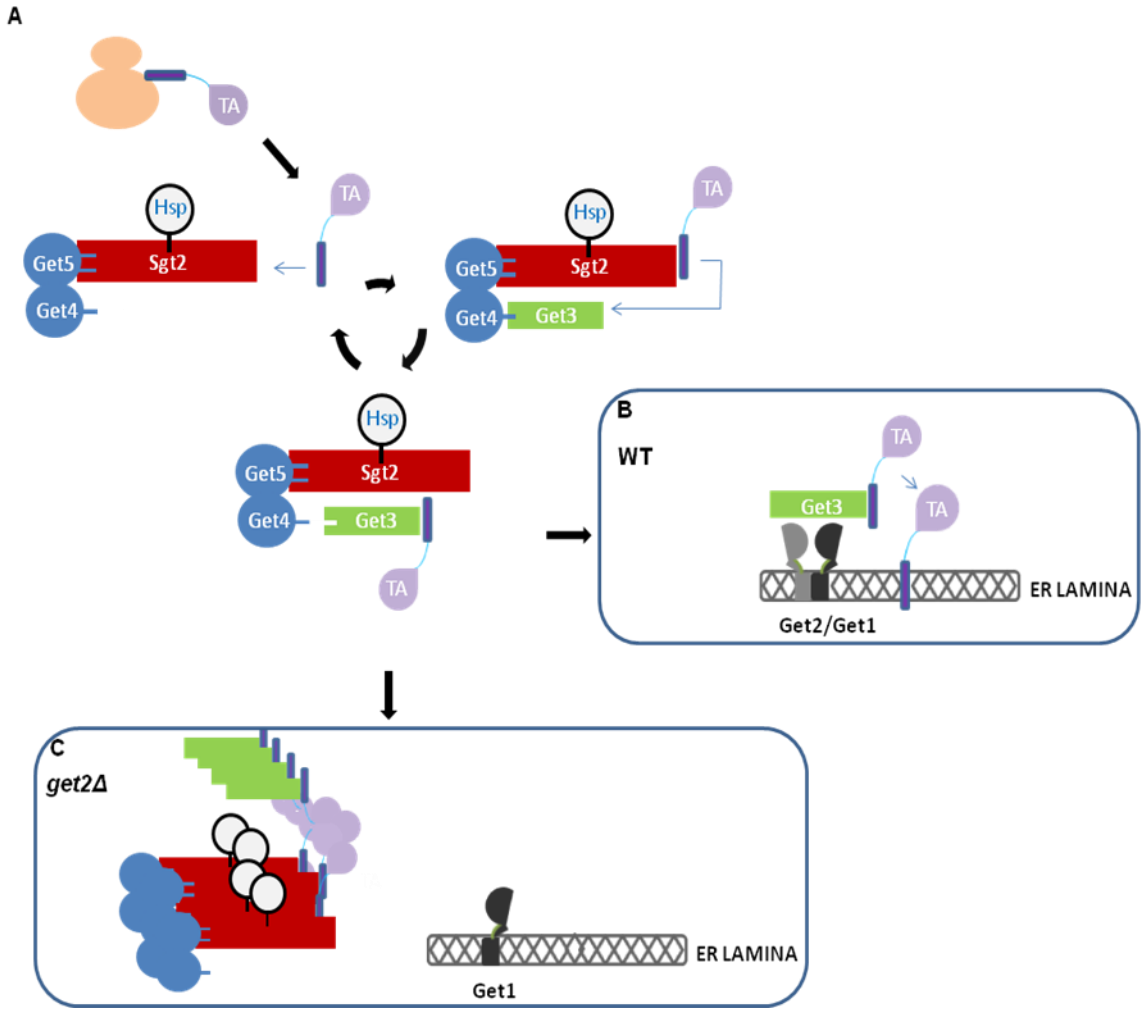


Figure 6 GET pathway

A) GET pathway is responsible for the targeted delivery of the TA proteins to the ER membrane. TA protein is recognized by the Sgt2-Get5-Get4 complex through a TA-binding domain in Sgt2. The TA protein is then handed off to Get3 which is stabilized in its closed conformation by Get5/Get4 for facilitating Get3-TA protein complex formation. B) The Get3-TA protein complex is recruited to the ER membrane by an interaction with Get1/Get2 complex. TA protein is then released and Get3 is recycled. C) In the absence of functional Get1/Get2complex TA-proteins fail to reach the ER membrane and form cytosolic aggregates. The cytosolic components of the GET pathway (Sgt2, Get3, Get4, and Get5) are sequestered by TA aggregates.

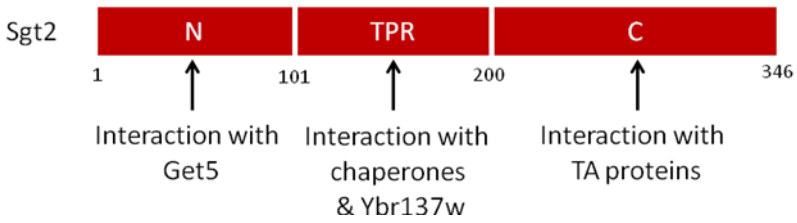


Figure 7 Structural and functional organization of the Sgt2 protein
N, TPR and C refer to Sgt2 N, Sgt2 TPR and Sgt2 C domains, respectively.

Data obtained previously in the Chernoff's lab showed that both mutation and deletion of *GET2* lead to the defect of [*PSI*$^+$] curing by excess Hsp104. Moreover, the deletions of components of the GET pathway, including *get1*Δ, *get2*Δ, *get3*Δ, *get4*Δ and *get5*Δ, all exhibit the similar defect. Last but not the least, *get2*Δ combined with *sgt2*Δ has a partial restoration to the defect. We believe that Sgt2 is the key mediator of the effect the defective GET pathway has on [*PSI*$^+$] curing.. The goal of this study is to investigate the genetic and physical interactions of Sgt2 protein with [*PSI*$^+$]-chaperone machinery.

2 Methods

2.1 Yeast strains

The only species of all the yeast used in this study is *Saccharomyces cerevisiae* (see Table 2 and Table 3). All strains, except the strains used in yeast two-hybrid assay, derived from GT81-1C and contain mutation *ade1-14* $_{UGA}$ that can be suppressed in [*PSI*$^{+}$] variants of the strains, and was used in experiments to detect the presence of the prion[39]. All of the used strains contain another prion, [*PIN*$^{+}$].

Table 2 Yeast strains used as the original source for this work

Strain	Genotype	Ref.
GT50	*MATα trp1-901 leu2-3,112 ura3-52 his3-Δ200 gal4Δ gal80Δ Gal2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-LaZ*	[40]
GT74	*MAT*a *trp1-901 leu2-3,112 ura3-52 his3-Δ200 gal4Δ gal80Δ Gal2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-LaZ*	[40]
GT81-1C	*MAT*a *ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52* [*PSI*$^{+}$][*PIN*$^{+}$]	[41]
GT159	*MAT*a *ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52* [*psi*$^{-}$][*PIN*$^{+}$]	[41]
GT1308	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 Δget4::hisMX* [*PSI*$^{+}$][*PIN*$^{+}$]	Not published
GT1309	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 get5Δ::hisMX* [*PSI*$^{+}$][*PIN*$^{+}$]	Not published

table # continued		
GT1449	*MAT*a *ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 sgt2::hisMX* [*PSI*$^+$][*PIN*$^+$]	Not published
GT1487-4A	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 get2Δ::hph sgt2Δ::hisMX* [*PSI*$^+$][*PIN*$^+$]	Not published
GT1507-1D	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 get3Δ::hisMX* [*PSI*$^+$][*PIN*$^+$]	Not published
GT1508-8C	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 get2Δ::hph* [*PSI*$^+$][*PIN*$^+$]	Not published
GT1509-1D	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 get1Δ::hisMX* [*PSI*$^+$][*PIN*$^+$]	Not published
GT1550	*MAT*a *ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 SGT2-HA::kanMX* [*PSI*$^+$][*PIN*$^+$]	Not published

Table 3 Yeast strains constructed in this work

Strain	Genotype
GT1589-4A	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 SGT2-HA::hisMX GET2//get2Δ::hph* [*PSI*$^+$][*PIN*$^+$]
GT1590-10C	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 SGT2-HA::kanMX GET1//get1Δ::hisMX [PSI*$^+$*][PIN*$^+$*]*
GT1602-10B	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 SGT2-HA::hisMX GET3//get3Δ::hisMX* [*PSI*$^+$][*PIN*$^+$]
GT1632	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 SGT2-HA::hisMX GET3//get4Δ::hisMX* [*PSI*$^+$][*PIN*$^+$]
GT1633	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 SGT2-HA::hisMX GET3//get5Δ::hisMX* [*PSI*$^+$][*PIN*$^+$]
GT1732	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 SGT2-HA::kanMX* [*psi*$^-$][*PIN*$^+$]
GT1733	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 SGT2-HA::kanMX get2Δ::hph* [*psi*$^-$][*PIN*$^+$]

table # continued	
GT1769	*MAT a ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 sgt2-R171A,R175A* [*PSI*$^+$][*PIN*$^+$]
GT1778	*MATα ade1-14SC his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 sgt2-R171A,R175A GET2//get2Δ::hph* [*PSI*$^+$][*PIN*$^+$]

Nine strains, GT1589-4A, GT1590-10C, GT1602-10B, GT1632, GT1633, GT1732, GT1733, GT1769, and GT1778 were constructed in this study. GT1589-4A, GT1590-10C, GT1602-10B, GT1632, GT1633 were created by mating GT1550 with GT1508-8C, GT1509-1D, GT1507-1D, GT1308 and GT1309 then tetrad dissected, respectively (see Chapter 2.7). GT1732 and GT1733 are [*psi*$^-$] derivatives of GT1550 and GT1589, respectively. [*psi*$^-$] strains were created by overexpressing Hsp104 (plasmid pLH105) in the original [*PSI*$^+$] strains. Transformants were streaked on YPD media for ~ 5days and several colonies were picked up to check the [*PSI*$^+$] status (on the media lacking adenine) and the plasmid presence (on media lacking leucine).

GT1769 is a *sgt2-R171A, R175A* mutation strain created by *Delitto Perfetto* technique (see Chapter 2.6). GT1778 was created by mating GT1769 with GT1589 then tetrad dissected. GT 1589, the strain with *SGT2* tagged by HA, was used to facilitate the selection of *sgt2* mutation in the tetrad analysis. Colonies were velveteened on hygromycin plate (200μg/ml) to check the *get2*Δ and G418 (500μg/ml) plate to check

sgt2 mutation (both confirmed by PCR).

2.2 Plasmids

All plasmids used in this study were produced in *Escherichia coli* strain DH5α (see Table 4 and Table 5).

Table 4 Plasmids used in this study

Plasmid	Protein	Type	Marker	Promoter	Ref.
pRS316-GAL	Vector	*CEN*	*URA3*	P_{GAL}	[40]
pACT2	Vector	*2μ*	*LEU2*	P_{ADH}	[40]
pSE1111	Snf4	*2μ*	*LEU2*	P_{ADH}	[40]
pSE1112	Snf1	*2μ*	*TRP1*	P_{ADH}	[40]
pGAL104-URA3	Hsp104	*CEN*	*URA3*	P_{GAL}	[40]
pAS1	Vector	*2μ*	*TRP1*	P_{ADH}	[40]
pGAL::SSA1	Ssa1	*CEN*	*URA3*	P_{GAL}	[42]
pLH101	Ssa1	*2μ*	*LEU2*	P_{SSA1}	[42]
pAS1-SUP35N	Sup35N	*2μ*	*TRP1*	P_{ADH}	[40]
pACT2-SUP35N	Sup35N	*2μ*	*LEU2*	P_{ADH}	[40]
pG4BD-0	Vector	*2μ*	*TRP1*	P_{ADH}	[43]
pG4BD-0-SUP35N	Sup35N	*2μ*	*TRP1*	P_{ADH}	[43]
pRS315	Vector	*CEN*	*LEU2*	P_{GPD}	[44]
pLH105	Hsp104	*CEN*	*LEU2*	P_{GPD}	[44]
pG4BD-SUP35	Sup35	*2μ*	*TRP1*	P_{ADH}	[43]
pG4BD-1-NMSc	Sup35NM	*2μ*	*TRP1*	P_{ADH}	Not published
pRS315/P_{GAL}-HSP104	Hsp104	*CEN*	*LEU2*	P_{GAL}	Not published
pRS316/P_{GPD}-HSP104	Hsp104	*CEN*	*URA3*	P_{GPD}	Not published
pCORE	Vector	*n/a*	*kanMX4/ URA3*	*n/a*	[45]

Table 5 Plasmids constructed in this study

Plasmid	Protein	Type	Marker	Promoter
pACT2-SGT2	Sgt2	*2μ*	*LEU2*	P_{ADH}
pAS1-SGT2	Sgt2	*2μ*	*TRP1*	P_{ADH}
pG4BD-SGT2	Sgt2	*2μ*	*TRP1*	P_{ADH}

Three plasmids, pACT2-SGT2, pAS1-SGT2, pG4BD-SGT2 were constructed in this study (see Figure 8). For the construction of pACT2-SGT2, the open reading frame (ORF) region of the SGT2 gene was PCR-amplified by using primers with the extensions containing the restriction sites for BamHI and XhoI (see Table 6) and then inserted into the polylinker of plasmids pACT2 cut by BamHI and XhoI. For construction of pAS1-SGT2, the ORF region of the SGT2 gene was PCR-amplified by using primers with the extensions containing the restriction sites for BamHI and XhoI and then inserted into the polylinker of plasmids pAS1 cut by BamHI and SalI. For construction of pG4BD-SGT2, the ORF region of the SGT2 gene was PCR-amplified by using primers with the extensions containing the restriction sites for BamHI and SmaI and then inserted into the polylinker of plasmids pG4BD cut by BamHI and SmaI.

Table 6 Primers used in this study

Lab#	Sequence	Used for
463	AAACGTACGACAAGAACAAG	Checking *GET3* deletion (Forward)
464	CACACACATACCATCGTATT	Checking *GET3* deletion (Reverse)
670	GATGGCTACTTGGGTTGAGC	Checking *GET2* deletion (Forward)
671	GATTTTTCACGAACTCATCGC	Checking *GET2* deletion (Reverse)
674	TGCACGTACCAACTACCTCCTG	Checking *GET1* deletion (Forward)
675	TTCAAAAGATTGGAGACGGAG	Checking *GET1* deletion (Reverse)
741	ATTGCCCTACGCTTAATCCC	Checking *SGT2* deletion (Forward)
742	GTCTTCTCCGAAAATCGACG	Checking *SGT2* deletion (Reverse)
754	GCTGCTCATTCATCCCTG	Sequencing *SGT2* ORF. Complementary to the middle part of *SGT2*. (Forward)
755	TTCTGGAACGGTTTTCTCC	Sequencing *SGT2* ORF. Complementary to the middle part of *SGT2* (Reverse)
766	TAAGGATCCTCTACTGTACGATGTCAG CATC	Amplification of *SGT2* ORF. *BamHI* site is introduced before start codon of *SGT2* (Forward)
767	ATGCTCGAGATTAAAGGCTTATTTCAG TCC	Amplification of *SGT2* ORF. *XhoI* site is introduced at 3' end of *SGT2* (Reverse)
863	CGTTAAGGTAACAAACAATGGG	Checking *GET4* deletion (Forward)
864	GGAGGCCCTTAATAGGTCTGC	Checking *GET4* deletion (Reverse)
865	GCACAGGAGAACATAGTTGGAG	Checking *GET5* deletion (Forward)
866	GAAAATAGTAAGCGCAACCG	Checking *GET5* deletion (Reverse)
876	TCAGCCCGGGATTGCTTGCTTGTTCTCA TTGTC	Amplification of *SGT2* ORF. *SmaI* site is introduced at 3' end of *SGT2* (Reverse)
958	GTCAAAGACGCTGAATCTGCAATTTCT ATTGACCCATCTTATTTCTCCTTACCAT TAAGTTGATC	Amplification of the CORE cassette and its introduction into *SGT2* gene (Forward)

table # continued		
959	AGGGCTTCTTCGGGTTTACCTTGCGCATATTTAGCAAAACCCAAAGAGCTCGTTTTCGACACTGG	Amplification of the CORE cassette and its introduction into *SGT2* gene (Reverse)
971	GACGCTGAATCTGCAATTTCTATTGACCCATCTTATTTCGCAGGCTACTCTGCATTGGGT	Amplification of the fragment to introduce substitutions R171A, R175A in *SGT2* gene (Forward)
972	CTTCTTCGGGTTTACCTTGCGCATATTTAGCAAAACCCAATGCAGAGTAGCCTGCGAAAT	Amplification of the fragment to introduce substitutions R171A, R175A in *SGT2* gene (Reverse)
989	CTTATTTCGCAGGCTACTCTGC	Checking R171A,R175A mutations in *SGT2* gene (Forward)

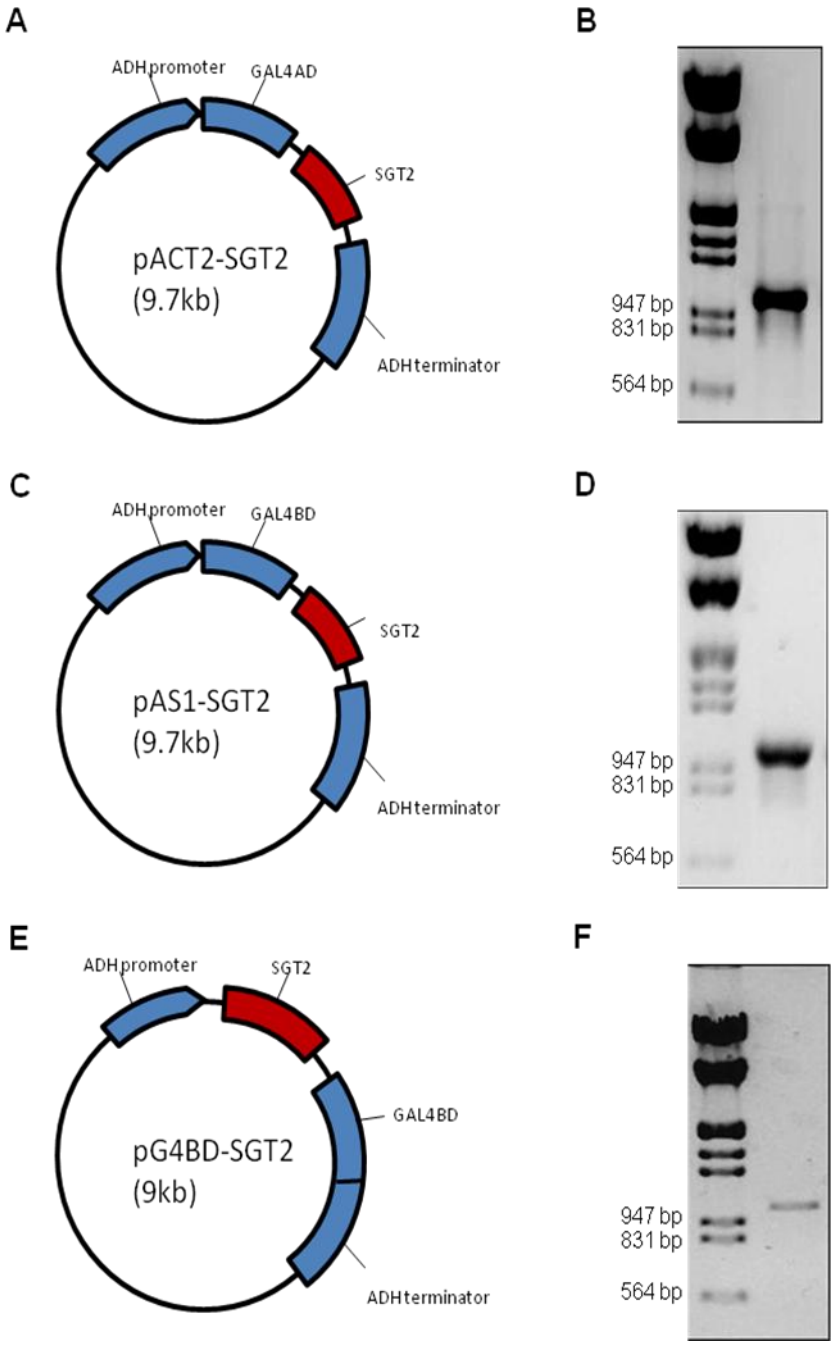


Figure 8 Plasmids containing Sgt2 constructed for yeast two-hybrid system

A) Full-length Sgt2 fused with Gal4 activation domain in pACT2 plasmid. B) PCR confirming the presence of Sgt2 in pACT2-SGT2. C) Full-length Sgt2 fused with Gal4 DNA binding domain in pAS1 plasmid. D) PCR confirming the presence of Sgt2 in pAS1-SGT2. E) Full-length Sgt2 fused with Gal4 DNA binding domain in pG4BD plasmid. F) PCR confirming the presence of Sgt2 in pG4BD-SGT2.

2.3 Antibodies

Antibodies to HA epitope were purchased from COVANCE and used in 1:5000 dilution. Antibodies to Sup35C were kindly provided Dr. David Bedwell and used in 1:5000 dilution. Antibodies to Ade2 were raised by Cocalico Biologicals (purified Ade2 protein was kindly provided by V. Alenin) and used in 1:2000 dilution. Antibodies to Ssa were generously provided by E.Craig and used in 1:30000 dilution. Secondary anti-mouse and anti-rabbit antibodies were purchased from Sigma-Aldridge and used in 1:12000 dilution.

2.4 Media and growth Conditions

Yeast cell cultures were all grown at 30℃. Standard yeast media and standard procedures for yeast cultivation were used. YPG media was used to eliminate petite colonies from all experimental evaluations. 2% galactose instead of glucose is used in YPGal media to induce P_{GAL} promoter. Liquid cultures were grown with a ~1/5 liquid/container volume ratio in a shaking incubator (225 rpm) [46].

2.5 Transformation of yeast by lithium acetate treatment

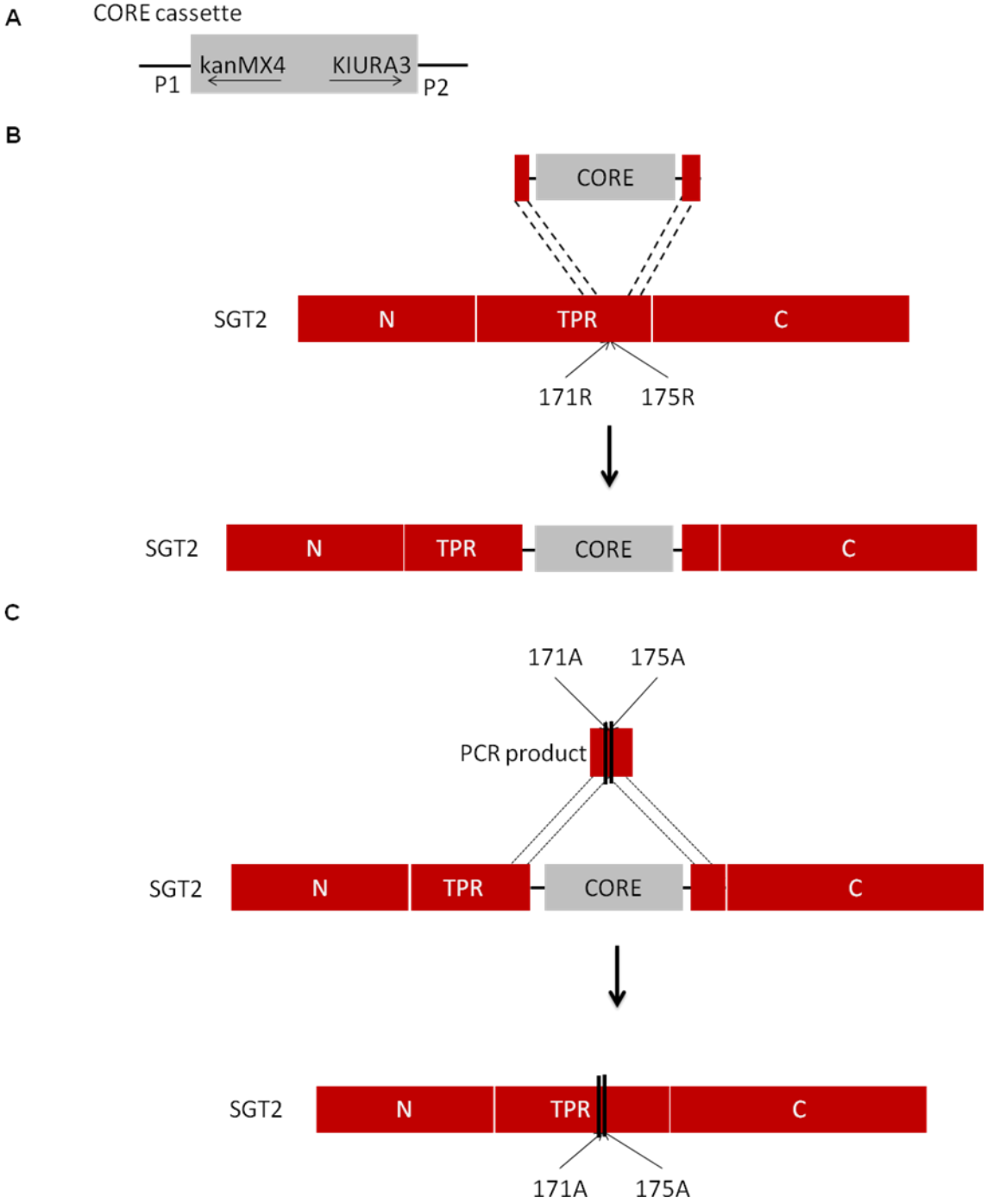
Yeast cells are pre-cultured in 5-10mls of YPD media overnight. Pre-cultures are diluted with fresh YPD media (5-7 fold) and incubated at 30℃ for ~5 hrs until they

re-enter the exponential growth phase. Cells are collected by centrifugation at 3,000 rpm for 5 mins, washed with H_20, and resuspended in 1mls of 100mM LiAc-TE for one transformation. Cells are incubated for an hour with shaking at 30℃. Afterwards, 20µg of carrier DNA and 1-10µg of transforming DNA are added. Tubes are incubated for 30 mins at 30℃. Following the incubation, 350µl per 50µl of cells of Li-Ac-PEG-TE (40% PEG 3350, 100mM LiAc, 10mM Tris-HCl, pH7.5, 1 mM EDTA) is added. After half hour of incubation at 30℃, the cells are heat shocked at 42℃ for 5 mins. Cells are then plated onto appropriate selective media[47].

2.6 Gene mutation by *Delitto Perfetto*

The *Delitto Perfetto* technique was used in this study to introduce double point mutation in the chromosomal copy of *SGT2* gene in the strain GT81-1C. For that the counterselectable reporter (CORE) cassette was amplified by using plasmid pCORE as a template (see Table 4). Pair of primers which are complementary to the P1, P2 (see Figure 9A) and also contain sequences identical to the flanking regions of the mutation site (see Table 6) were used for the PCR. PCR amplification of CORE cassette was performed with high yield in a final volume of 100µl by using Taq DNA polymerase (NEB) and a following program: 5 mins at 95℃, 32 cycles of 30secs at 95℃, 30secs at 55℃, and 4 mins at 72℃ by using primers 958, 959 (see Table 6). PCR product was

transformed into the GT81-1C strain (see Figure 9B). Clones with the integrated CORE cassette were selected on -Ura media first and then double checked by their ability to grow on G418 media. On the next step cells with integrated CORE cassette were transformed with the PCR fragment identical to a region of Sgt2 with the designed point mutations (see Figure 9C). This transformation led to the excision of the CORE cassette and introduction of the mutation *sgt2-R171A, R175A*, which corresponds to substitution of arginines in positions aa171 & 175 by alanines in Sgt2 protein. PCR amplification of this fragment was performed in a final volume of 50µl by using Deep Vent DNA polymerase (NEB) and a following program: 5 mins at 95℃, 6 cycles of 10secs at 95℃, 30secs at 55℃, and 10secs at 72℃ by using primers 971 and 972 (see Table 6). Counterselection against *KlURA3* marker was performed on 5-FOA media and followed by testing for simultaneous loss of the *kanMX4*. The presence of the point mutations was confirmed by PCR with the allele-specific forward primer complementary to the mutation site (see Table 6) and a reverse primer complementary to the sequence downstream of the *SGT2* ORF(see Figure 9D). PCR amplification of this fragment was performed with high yield in a final volume of 20µl by using Taq DNA polymerase and a following program: 5 min at 95℃, 30 cycles of 30secs at 95℃, 30secs at 55℃, and 90secs at 72℃[45].


A
CORE cassette
kanMX4
KIURA3
P1
P2
B
CORE
SGT2
N
TPR
C
171R
175R
SGT2
N
TPR
CORE
C
C
171A
175A
PCR product
SGT2
N
TPR
CORE
C
SGT2
N
TPR
C
171A
175A

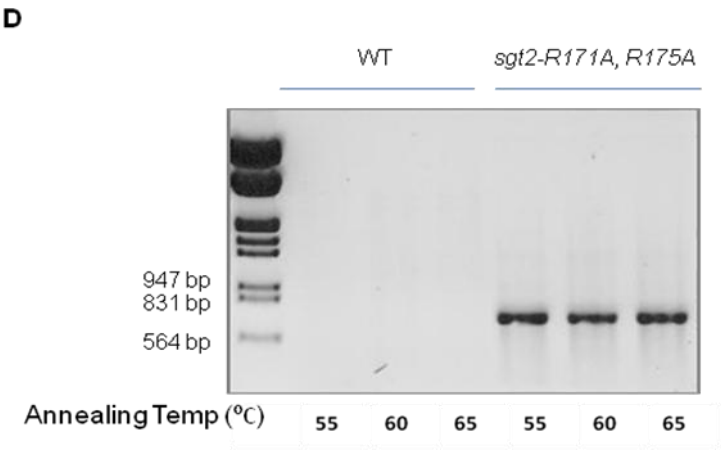


Figure 9 Introduction of point mutations into SGT2 gene by Delitto Perfetto
A) The CORE cassette for integrating two yeast markers nearby the mutation site (aa171 & 175) of Sgt2. The cassette was amplified by using plasmid pCORE as a template (Table 3) and a pair of primers which are complementary to the P1, P2 and also contain sequences identical to the flanking regions of the mutation site (Table 5). B) PCR product was transformed into the wild type strain. Integration of the cassette occurs by homologous recombination within the flanking regions of the mutation site. Clones with the integrated CORE cassette were selected on -Ura media first and then double checked by their ability to grow on G418 media. C) Transformation of the cells with a designed PCR fragment leads to the excision of CORE cassette and substitution of arginines in positions aa171 & 175 by alanines. Counterselection against *KlURA3* marker was performed on 5-FOA mediam and followed by testing for simultaneous loss of the *kanMX4*. D) The presence of the point mutations was confirmed by PCR with the allele-specific forward primer complementary to the mutation site (Table 5) and a reverse primer complementary to the sequence downstream of the *SGT2* ORF.

2.7 Tetrad dissection and analysis

Two haploid strains with opposite mating types were crossed on YPD and incubated for one day. Afterwards, the diploids were patched on sporulation media and incubated for more than 3 days until a sufficient number of cells have sporulated. Cells are then subjected to 40µl zymolase (0.5 mg/ml) for 2 minutes at 37^{o}C to remove cell wall and weaken the ascus. Then a small portion of the cells are placed on YPD and dissected

into their individual spores using a micromanipulator (The Singer MSM System 300). The spores are incubated at 30^{o}C until colonies are formed.

2.8 Yeast two hybrid system

This assay (see Figure 10) was performed in two variants: in the haploid strain and in the diploid strain[48 49].

In the haploid strains, double plasmid transformation, with both Prey and Bait, was performed in GT74 strain in all pair-vise combinations. Eight colonies were selected from each transformation plates and were patched onto media lacking leucine and tryptophan. These plates were cultured for ~2 days, and were then replica-plated onto YPD, media lacking adenine, media lacking both leucine and tryptophan, and YPG. The protein interaction was estimated by examining the growth on media lacking adenine after ~4 days.

In diploid strains, strain GT74 was transformed by pACT2-based plasmids (Prey) and strain GT50 was transformed by pG4BD-based plasmids (Bait) in all pair-vise combinations. Two colonies were selected from each transformation plates and were patched onto media lacking leucine or tryptophan. These plates were cultured for ~2

days, and then cells were patched onto media lacking both leucine and tryptophan for mating. Each two colonies of GT74 transformants were mated with each two colonies of GT50 transformants, thus giving four diploid strains for every Prey-Bait combination. Diploids were selected on media lacking both leucine and tryptophan and were then replica-plated on YPD, media lacking adenine, media lacking both leucine and tryptophan, and YPG. The protein interaction was estimated by examining the growth on media lacking adenine after ~ 5 days.

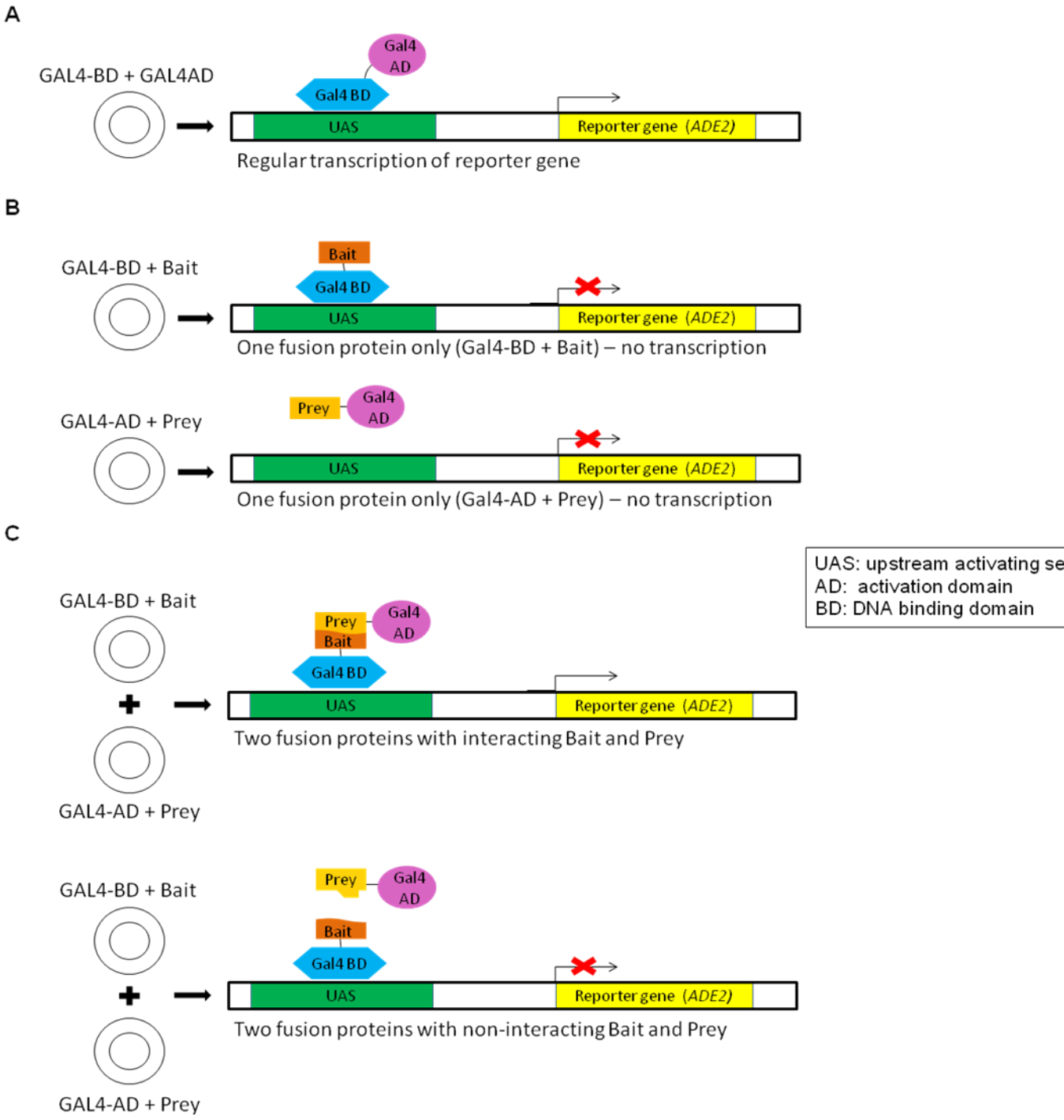


Figure 10 Overview of yeast two hybrid (Y2H) assay

A) Gal4 transcription factor consists of two domains (BD and AD) which are both essential for transcription of the reporter gene (*ADE2*). B) To check if two proteins (called Bait and Prey here) interact with each other two fusion constructs are prepared: Gal4BD+Bait and Gal4AD+Prey. None of them is sufficient for initiating the transcription of *ADE2* alone. **C)** When both fusion proteins are produced and Bait interacts with Prey, the transcription of *ADE2* begins.

2.9 Yeast protein isolation

All the cell cultures were grown at 30℃ till the OD_{600} reached 0.4-0.5. Cell pellets were collected by centrifugation at 3000 rpm for 5 mins. To isolate proteins, cell pellets were resuspended in lysis buffer (50mM Tris-Cl pH 7.5, 200mM NaCl, 1 % Triton X-100, 0.1%SDS, 1mM EDTA, 1mM PMSF in Complete^{TM} protease inhibitor cocktail (Roche). Acid-washed glass beads were added and the solution was vortexed for 1 min for 5 times with 1 min on ice between vortexing. To collect total lysates, cell debris was removed by centrifugation at 6000 rpm for 2 min. The protein concentration was estimated by Bradford assay.

2.10 SDS-PAGE and Western-blotting

Protein was mixed with 1/3 volume of 4X loading buffer (600Mm Tris-Cl pH6.8, 12% SDS, 40% glycerol 12% 2-mercaptoethanol and 0.6% bromophenol blue) and boiled for 5 min. After boiling, the protein sample was loaded on the 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed at 100 volts for 2 hour. After the electrophoresis, proteins were transferred from the gels to a PVDF membrane (Immobilon-P Membrane, Millipore) using the tank electroblotting system (Bio-Rad). Before transfer, the PVDF was incubated first in methanol and then in transfer buffer (10% methanol, 10mM CAPS, pH11.0). Also, the

gel was incubated in transfer buffer before the transfer. The transfer was performed at 4℃, 275mA for 45 mins. Appropriate primary antibodies then were used to perform Western-blotting analysis. Then the membranes were probed with appropriate secondary antibodies and developed according to the Amersham ECL detection system protocols (250mM luminal, 90mM coumaric acid, 0.03% H_2O_2, 0.1 m Tris pH8.5).

2.11 Co-Immunoprecipitation of proteins interacting with Sgt2

All the cell cultures were grown at 30℃ till the OD_{600} reached 0.4-0.5. Cell pellets were collected by centrifugation at 3000 rpm for 5 mins. To isolate proteins, cell pellets were resuspended in lysis buffer (75mM KCl, 25mM Tris, 0.5% Triton X-100, pH 7.0, 1mM PMSF in Complete^{TM} protease inhibitor cocktail (Roche). Acid-washed glass beads were added and the solution was vortexed for 1 min for 5 times with 1 min on ice between vortexing. To collect total lysates, cell debris was removed by centrifugation at 1000 rpm for 10 min at 4℃. The protein concentration was estimated by Bradford assay.

The fallowing steps (see Figure 12A) were performed at 4℃. Protein lysate was first incubated with HA antibody (Ab) overnight. Then the Ab-protein complex was captured by 2 hours incubation with protein-A agarose. . Afterwards, the resin was

washed 3 times by PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4 1.47mM KH_2PO_4 pH 7.0). Wash buffer was removed by centrifugation at 1000 rpm for 2 mins. Identification of proteins in the pellet was performed by Western-blotting.

2.12 Curing of [*PSI*$^+$] by Hsp104 overproduction

2.12.1 Qualitative curing of [*PSI*$^+$] by Hsp104 expressed from GPD promoter

To overproduce Hsp104, strains were transformed with the plasmid containing *HSP104* gene under P_{GPD} promoter. Four colonies were selected for each transformation and were patched onto synthetic media according to the yeast maker of that plasmid. Plates were incubated for ~ 3 days, and then were replica-plated onto YPD, the relative synthetic media, the relative synthetic media also lacking adenine, and YPG. YPD and the relative synthetic media were used to control growth rate and plasmid maintaining. YPG media was used to eliminate petite clones from experimental evaluation. The curing was estimated by examining the growth on that relative synthetic media also lacking adenine after ~5 days.

2.12.2 Qualitative curing of [*PSI*$^+$]by Hsp104 expressed from GAL promoter

To overproduce Hsp104, strains were transformed with the plasmid containing *HSP104* gene under P_{GAL} promoter. Four colonies were selected for each transformation and

were patched onto synthetic media relative to the yeast maker of that plasmid. Plates were incubated for ~ 3 days, and then were velveteened onto YPGal media and incubated for one day. Cells were then repatached on the relative synthetic media and incubated for 2 days. These plates were then velveteened on YPGal again (2^{nd} round induction) and incubated for one day. Cells were then repatached on the relative synthetic media. These plates were cultured for ~ 3 days, and then were replica-plated onto YPD, the relative synthetic media, the relative synthetic media also lacking adenine, and YPG. YPD and the relative synthetic media were used to control growth rate and plasmid maintaining. YPG media was used to eliminate petite colonies from experimental evaluation. The curing was estimated by examining the growth on that relative synthetic media also lacking adenine after ~5 days.

3 Results

3.1 The physical interaction between Sgt2 and Sup35

The effect of *sgt2*Δ on [*PSI*$^+$] curing in the strain with defective GET system could be explained by three different mechanisms. First, Sgt2 can potentially interact with [*PSI*$^+$] aggregates and change its properties. Second, Sgt2 might be needed for chaperones to interact with [*PSI*$^+$]. Third, Sgt2 might prevent chaperones from interaction with [*PSI*$^+$] by retargeting them into a different pathway.

The first two models suggest a direct or indirect interaction of Sgt2 with [*PSI*$^+$] . If one of these two models is correct we would expect Sgt2 to interact with Sup35.

3.1.1 Interaction between Sgt2 and Sup35 in yeast two-hybrid system

To check if Sgt2 can interact with Sup35 we used yeast two hybrid (Y2H) system to test the interaction between Sgt2 and full length Sup35, and its truncated versions (N domain and NM domain). This assay was performed in two variants: in the haploid strain and in the diploid strain (see Chapter 2.9). Comparison of the interaction in both variants of the assay will allow us to decrease the probability of false positive or false negative results.

In the haploid strains, double plasmid transformation, with both Prey and Bait, was performed in GT74 strain in all pair-vise combinations. Eight colonies were selected from each transformation plates and were patched onto media lacking leucine and tryptophan. These plates were cultured for ~2 days, and were then replica-plated onto YPD, media lacking adenine, media lacking both leucine and tryptophan, and YPG. The protein interaction was estimated by examining the growth on media lacking adenine after ~4 days.

The results (see Figure11A and Table 7) suggest a relatively weak but detectable interaction between full-length Sgt2 (as Bait) and N-terminal domain of Sup35 (as Prey). If Sup35 was used as Bait, the results demonstrated interaction of Sup35 or Sup35N with all Prey constructs (including empty vector as negative control). No interaction was detected for Sup35NM as Bait with any other constructs including positive control (Sla1C).

Table 7 Results of Y2H experiment in haploid

AD (pACT2) \ BD (pG4BD)	Sgt2	Empty	Sup35N	Sup35	Sup35NM
Sgt2	+	-	+	+	-
Empty	-	-	+	+ -	-
Sla1C	-	-	+	+ -	-
Sup35N	+	-	+	+	-

+	=	Stronger growth
+ -	=	Normal growth
- +	=	Weaker growth
-	=	No growth

To test the interaction between Sgt2 and Sup35 in diploid strains, strain GT74 was transformed by pACT2-based plasmids (Prey) and strain GT50 was transformed by pG4BD-based plasmids (Bait) in all pair-vise combinations. Two colonies were selected from each transformation plates and were patched onto media lacking leucine or tryptophan. These plates were cultured for ~2 days, and then cells were patched onto media lacking both leucine and tryptophan for mating. Each two colonies of GT74 transformants were mated with each two colonies of GT50 transformants, thus giving four diploid strains for every Prey-Bait combination. Diploids were selected on media lacking both leucine and tryptophan and were then replica-plated on YPD,

media lacking adenine, media lacking both leucine and tryptophan, and YPG. The protein interaction was estimated by examining the growth on media lacking adenine after ~ 5 days.

The growth of diploids containing Sgt2 (as Bait) and Sup35N (as Prey) (see Figure 11B and Table 8) suggests the interaction between these two proteins. In case if Sup35N served as Bait, the results again demonstrated interaction with all used Prey constructs (including empty vector as negative control).

Table 8 Results of Y2H experiment in diploid

AD (pACT2) \ BD (pAS1)	Sgt2	Snf1	Empty	Sup35N
Sgt2	-	-	-	+ -
Empty	-	-	-	- +
Snf4	-	+	-	+ -
Sup35N	- +	-	-	+ -

+	=	Stronger growth
+ -	=	Normal growth
- +	=	Weaker growth
-	=	No growth

The growth of the strains carrying both Sgt2 and Sup35N constructs is much weaker compared to the growth of the strains with Snf1 and Snf4 used as a positive control, that suggests that the interaction between Sgt2 and Sup35 is relatively weak or transient. The similar results for Sgt2 interaction with Sup35N in both diploid and haploid strains in Y2H strongly suggest that this interaction is genuine. However, the Sgt2 self- interaction could be only detected in the haploid strain.

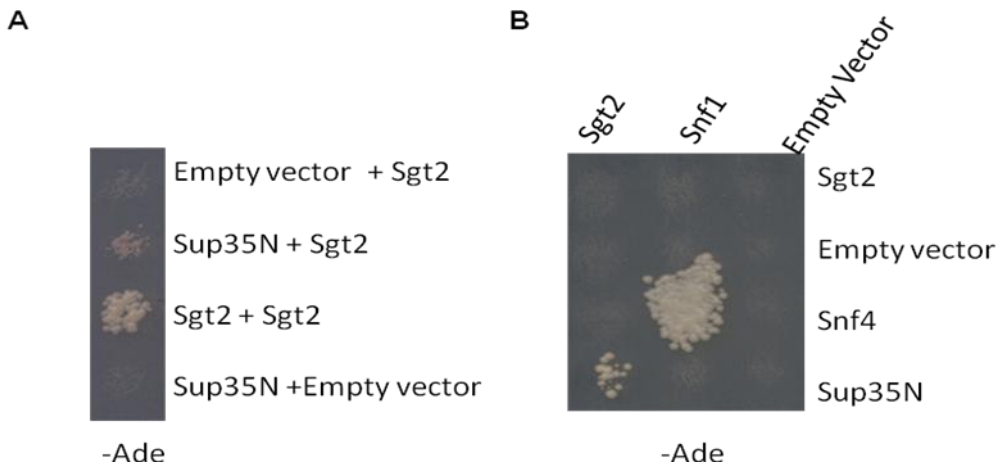


Figure 11 Interaction between Sgt2 and Sup35 in Y2H

A) Double plasmids transformation was performed in GT74 strain. The transformants were patched on -Trp-Leu media and replica plated to -Ade media. Image was taken after ~4 days of incubation. The growth of the strains indicates interaction between respective proteins. B) GT74 was transformed by pACT2-based plasmids and GT50 was transformed by pG4BD-based plasmids in all pair-vise combinations. The transformants of each strain were patched on –Trp-Leu medium for mating. Diploids then picked and patched on -Trp-Leu media and replica plated to -Ade media. Image was taken after ~5 days of incubation. The growth of the strains indicates interaction between respective proteins.

3.1.2 Interaction between Sgt2 and Sup35 shown by co-immunoprecipitation

To verify the data obtained in Y2H assay we examined the interaction between Sgt2 protein and Sup35 protein by using the co-immunoprecipitation (Co-IP) method. In this experiment, both [*PSI*$^+$] and [*psi*$^-$] strains were examined and compared. We performed this assay in both wild type and *get2*Δ strains to check if defect of the GET pathway changes the efficiency of this interaction.

Since the interaction between Sgt2 and Sup35 is relatively weak, we optimized the Co-IP protocol used in this study to detect even very low amounts of interacting proteins (see Chapter 2.11). A low-salt lysis buffer was used to avoid the disruption of interactions while preparing the protein lysate and a very "soft" wash buffer was used to clean the resin without breaking the specific interaction between proteins. A low speed centrifugation is required from the very first step of the resin cleaning to prevent a contamination of the samples with precipitated [*PSI*$^+$] aggregates.

The results (see Figure 12B) show that Sup35 does interact with Sgt2 in both [*PSI*$^+$] and [*psi*$^-$] strains, supporting the previous results obtained in Y2H assay that native Sup35 interacts with Sgt2. Also these results show that not only the native form but also the prion form of Sup35 interacts with Sgt2. We observed that the amount of Sup35 interacting with Sgt2 in [*PSI*$^+$] strains is higher than in [*psi*$^-$]. This is an expected effect that matches either two mechanisms we proposed in the case if Sgt2 interacts with Sup35. According to these hypotheses, [*PSI*$^+$]

aggregates will sequester Sgt2 by its direct or indirect interaction with the prion form of Sup35.

By comparing the results from wild type and *get2*Δ strains, the portion of Sup35 interacting with Sgt2 in *get2*Δ strain is smaller than in wild type strain. Moreover, Sgt2 has a higher expression in *get2*Δ strain than in wild type (also see Figure 13 and Chapter 3.2.1). A possible reason for the decreased amount of the Sup35 interacting with Sgt2 is that part of Sgt2 is sequestered by the TA protein aggregates caused by defect in the GET pathway in *get2*Δ strain. Aggregation of Sgt2 prevents it interaction with other proteins including Sup35.

To make sure that Sup35 expression maintains at the same level in each strain, the immunoblotting of the same protein lysates used for Co-IP was done for comparing the Sup35 levels. The result showed that the expressions of Sup35 are approximately equal in all strains which means the portion of Sup35 interacting with Sgt2 depends on the levels of Sgt2 and accessibility of Sup35 and Sgt2 for interaction.

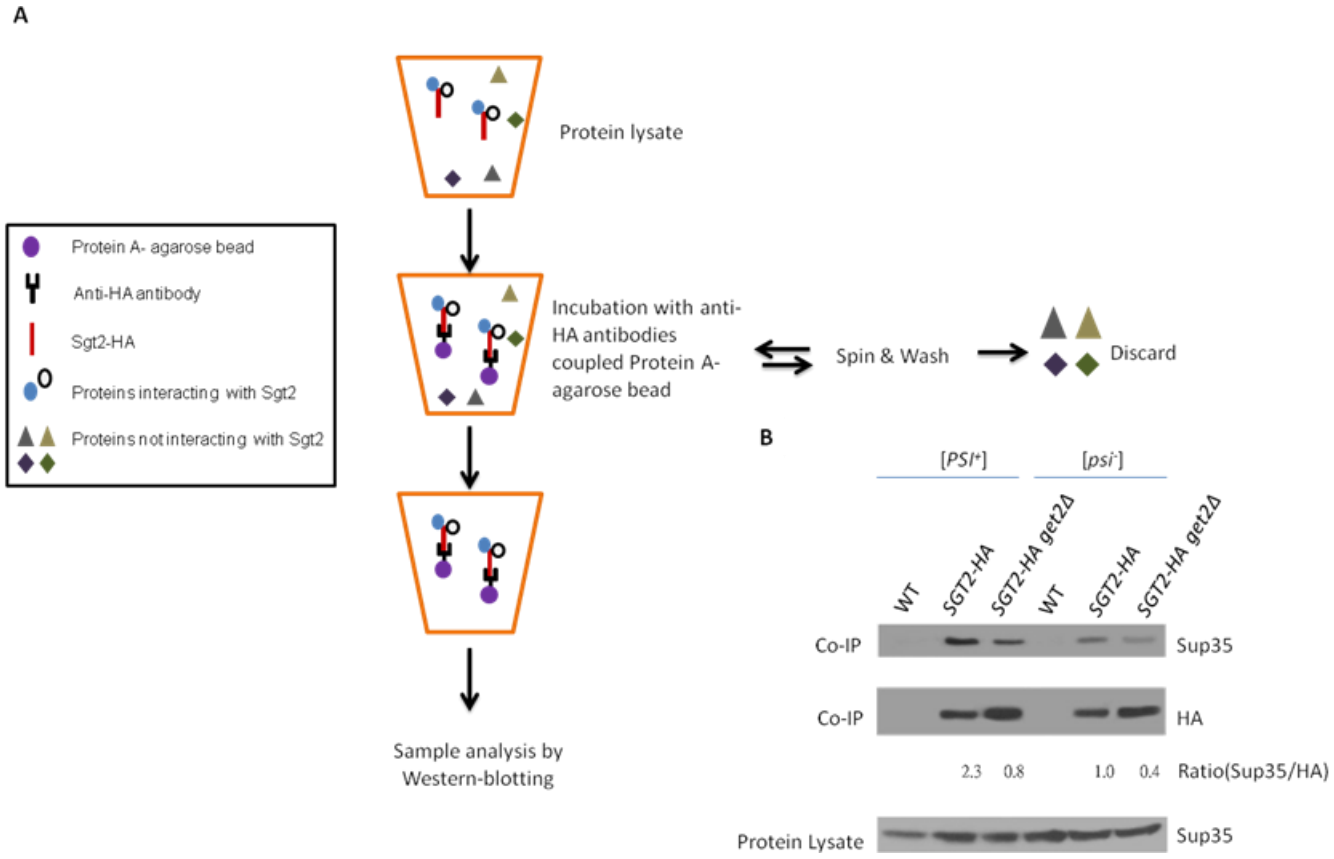


Figure 12 Co-immunoprecipitation of Sup35 with Sgt2

A) The overview of co-immunoprecipitation (Co-IP) of the proteins interacting with HA-tagged Sgt2. HA antibodies (Ab) are added to the protein lysate. Then the Ab-protein complex is pelleted using protein-A agarose. Sgt2-interacting proteins will be precipitated with Ab-Sgt2 complex. Identification of proteins in the pellet was performed by Western-blotting. B) The Interaction between Sgt2 and Sup35 detected by Co-IP. The result shows that smaller portion of Sup35 interacts with Sgt2 in *get2*Δ strains compared to wild type strains, and similar results are shown in both [*PSI*$^{+}$] and [*psi*$^{-}$] cells. The protein lysate controls at the bottom panel show the approximately equal level of Sup35 in each strain.

3.2 Sgt2 levels depend on the functional GET pathway and [*PSI*$^{+}$] status of the strain

The expression of chaperones and co-chaperones changes in response to the aggregation of the misfolded proteins accumulating in the cytoplasm. We addressed

the question if Sgt2 (described as a potential co-chaperone) levels depend on the presence of GET/TA aggregates and/or [*PSI*$^{+}$] aggregates in the cells.

3.2.1 Sgt2 levels are higher in the strains with defective GET pathway

The Co-IP experiments (see Figure 12) suggest that Sgt2 has a higher expression level in *get2*Δ than in wild type strain. We addressed the question if Sgt2 levels are also increased in other *get*Δ strains or it is specific for *get2*Δ only.

Protein lysates from wild type, *get1*Δ, *get2*Δ, *get3*Δ, *get4*Δ and *get5*Δ strains were isolated and compared by the Sgt2 levels using Western blotting (see Chapter 2.10). The results (see Figure 13) showed that there is more Sgt2 in any *get*Δ stain compared to the wild type, especially in *get2*Δ. It suggests that high Sgt2 expression level is a result of the defect in the GET pathway regardless of the GET gene deleted. Still, deletions of individual GET components have different levels of Sgt2 overproduction that might reflect a difference in the interaction between GET proteins with Sgt2.

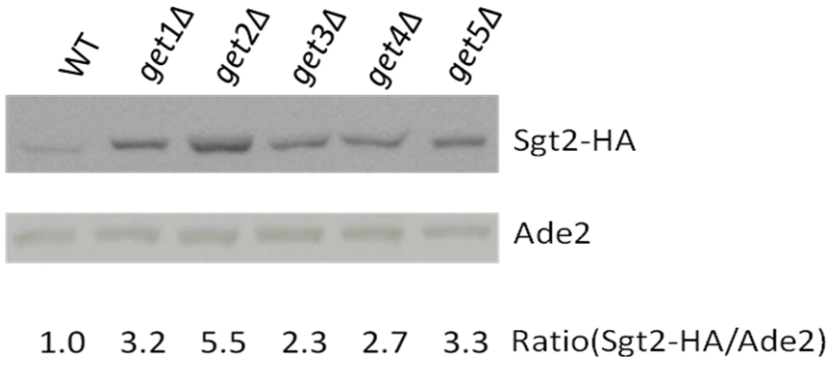


Figure 13 Sgt2 levels are higher in the strains with defective GET pathway
Wild type, *get1*Δ, *get2*Δ, *get3*Δ, *get4*Δ and *get5*Δ strains were compared by the Sgt2 levels. Sgt2 was fused to the HA tag and detected by hybridization with anti-HA antibodies. Ade2 was used as a loading control. The result shows that there is more Sgt2 is in the cell in any *get*Δ stain than in wild type, especially in *get2*Δ.

3.2.2 Sgt2 levels depend of [*PSI*$^+$] status of the strains

[*PSI*$^+$] and [*psi*$^-$] variants of *get2*Δ and wild type strains were also compared by the Sgt2 levels using the standard Western-blotting protocol (see Chapter 2.10). The result (see Figure14) showed that levels of Sgt2 are higher in [*PSI*$^+$] cells compared to the isogenic [*psi*$^-$] cells regardless of the status of the GET pathway. It suggests that any aggregate appears in the cell could possibly induce Sgt2 overproduction.

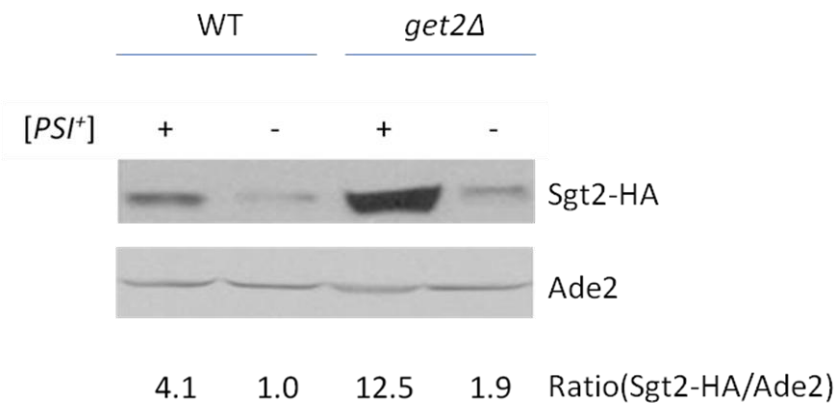


Figure 14 Sgt2 levels depend on [PSI+] status of the strains

[*PSI*$^+$] and [*psi*$^-$] variants of *get2*Δ and wild type strains were compared by the Sgt2 levels. Sgt2 was fused to the HA tag and detected by hybridization with anti-HA antibodies. Ade2 was used as a loading control. [*PSI*$^+$] cells generate more Sgt2 than [*psi*$^-$] cells, also more Sgt2 is produced in *get2*Δ strain compared to wild type in both [*PSI*$^+$] and [*psi*$^-$] backgrounds.

3.3 Sgt2 indirectly assists Ssa in protection of [*PSI*$^+$] from the curing

Data obtained previously in the Chernoff's lab showed that overproduction of Sgt2 itself does not cause any defect in [*PSI*$^+$] curing. There must be other protein(s), involved in this process to protect [*PSI*$^+$] from curing. The most likely candidates for the protein causing [*PSI*$^+$] curing defect are chaperones Hsp70-Ssa that are known to interact with Sgt2 and were shown to play a significant role in protecting [*PSI*$^+$] from Hsp104.

3.3.1 Sgt2 overproduction increases the effect of Ssa1 on [*PSI*$^+$] protection

The previous results obtained in the Chernoff's lab show that depletion of Sgt2 greatly

reduces the protective effect of Ssa1 overproduction of [*PSI*$^+$] curing. We addressed the question if physical interaction between Sgt2 and Ssa is needed for [*PSI*$^+$] protection from curing. For that we used site-directed mutagenesis to substitute arginines in the positions 171 and 175 in Sgt2 to alanines. This double mutation is known to disrupt completely the interaction between Sgt2 and chaperones Hsp104 and Hsp70 (Ssa). Thus, protein Sgt2-R171A, R175A cannot interact with chaperones through its TPR domain but still has functional N and C terminal domains.

We tested the [*PSI*$^+$] curing efficiency in the strain bearing chromosomal mutation *sgt2-R171A, R175A* in comparison t*o sgt2*Δ and wild-type strains with or without overproduction of Ssa1 in the presence of excess Hsp104.

For overproduction of both Ssa1 and Hsp104, th*e sgt2*Δ*, sgt2-R171A, R175A*, and wild type strains were transformed by two plasmids: one for Ssa1 overproduction and the other for Hsp104 overproduction. Experiment was performed in two independent sets different by the promoter that controll*d* the expression of *HSP104* an*d SSA1*. In the first set pRS316/P_{GPD}-HSP104 plasmid (with *URA3* marker) contained *HSP104* gene under the strong constitutiv*e* P_{GPD} promoter, and pLH101 multicopy *2μ* plasmid (with *LEU2* marker) had *SSA1* gene under its own promoter. In the second set,

pRS316/P_{GAL}-HSP104 (with *LEU2* marker) and pGAL::SSA1 (with *URA3* marker) plasmids contain*ed HSP104* and *SSA1* genes, respectively, under galactose inducible promoter P_{GAL}.

Four colonies were selected from each transformation and were patched onto media lacking leucine and uracil. For the first set plates were incubated for ~ 3 days, and then were replica-plated onto YPD, media lacking leucine and uracil, media lacking adenine and leucine and uracil, and YPG. The curing was estimated by examining the growth on media lacking adenine and leucine & uracil after ~5 days. For P_{GAL} set plates were incubated for ~ 3 days, and then were velveteened onto YPGal media and incubated for one day. Cells were then repatached on media lacking leucine and uracil and incubated for 2 days. These plates were then velveteened onto YPGal again (2^{nd} round of induction) and incubated for one day. Cells were then repatached on media lacking leucine and uracil. These plates were cultured for ~ 3 days, and were then replica-plated onto YPD, media lacking leucine and uracil, and media lacking adenine and leucine and uracil. The curing was estimated by examining the growth on media lacking adenine and leucine and uracil after ~5 days.

In both sets of the experiment we observed that Ssa1 overproduction protects [PSI^{+}]

from curing by excess Hsp104 in the *sgt2-R171A, R175A* strain to the extent of the same effect observed in the wild type strain(see Figure 15). Meanwhile, in the *sgt2*Δ strain protection was not efficient. Taken together, our results prove that Sgt2 assists Ssa in the protection of [PSI^+] from curing by excess Hsp104. Moreover, this effect does not require the direct interaction between Sgt2 and Ssa**.**

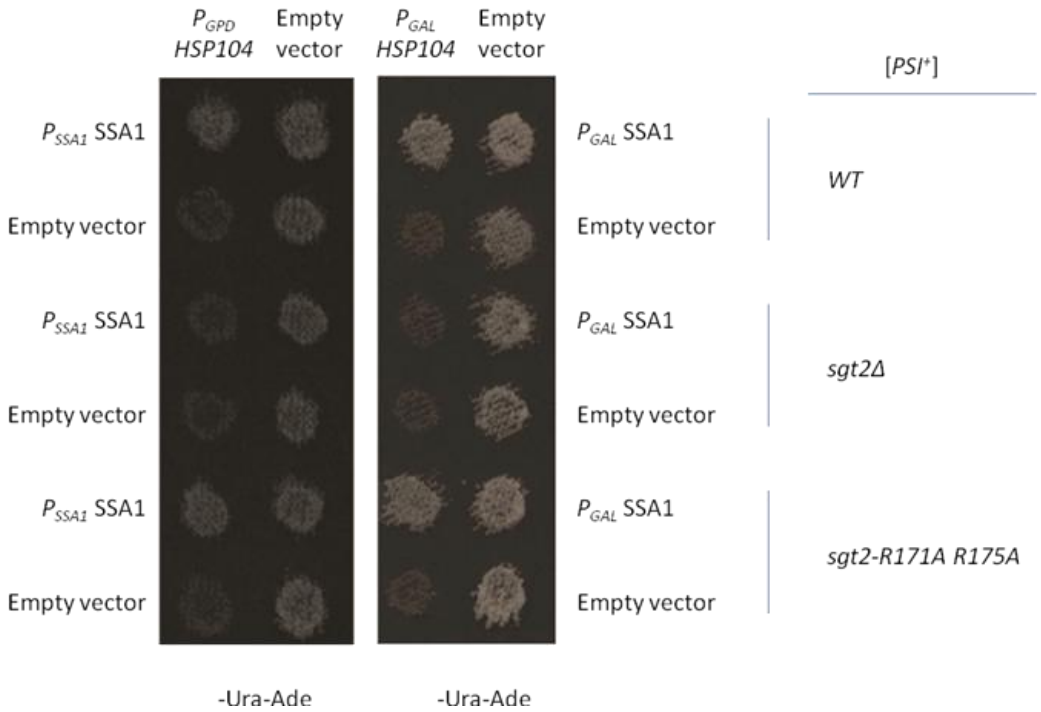


Figure 15 The effect of disrupting the interaction between Sgt2 and chaperones on the protection of [PSI$^+$] curing by overexpressed Ssa

Comparison of the efficiency of [PSI^+] protection by overexpressed Ssa in wild type strain and strains lacking Sgt2 or having mutant form of Sgt2. Expression of *HSP104* was controlled by either strong constitutive (P_{GPD}) or strong inducible (P_{GAL}) promoters. The results show that *sgt2-R171A, R175A* has no effect on protection of [PSI^+] curing.

3.3.2 Protection of [*PSI*$^+$] from curing in *get2*Δ strain depends on levels of Sgt2 and Ssa

We suggested that the defect of [*PSI*$^+$] curing in the strains with the defect of the GET pathway is observed due to both Sgt2 and Ssa overproduction. If this hypothesis is correct we should see the [*PSI*$^+$] protection in the strains with *get2*Δ having both Sgt2 and Ssa overproduction. According to the unpublished data obtained in the Chernoff's lab, in the strain with defect in the GET pathway levels of both Sgt2 and Ssa are increased. We predicted that in *get2*Δ strain Sgt2 will be overproduced even if Sgt2 has point mutation preventing it from interactions with chaperones and the defect of [*PSI*$^+$] curing will be observed. To test this prediction we constructed a strain that combines *get2*Δ and *sgt2-R171A, R175A* mutation. Six strains, *get2Δ*, *sgt2*Δ, *get2Δsgt2*Δ, *sgt2-R171A, R175A* and *get2Δ sgt2-R171A, R175A* alone with the wild type control were compared by the efficiency of [*PSI*$^+$] curing and Ssa levels.

To verify our hypothesis, the first thing needed to be confirmed is that the *get2Δ sgt2-R171A, R175A* strain has high levels of Ssa. Protein lysates from six strains mentioned above were isolated and compared by the Ssa1 levels by using the standard Western-blotting protocol (see Chapter 2.10).

Results (Fig.16) show that all *get2Δ* strains regardless of the *SGT2* state (*get2Δ*, *get2Δ*

sgt2Δ, and *get2Δ sgt2-R171A, R175A)* have excess Ssa1 comparing to *GET2* strains (wild type, *sgt2Δ*, and *sgt2-R171A, R175A*). As we expected, Ssa was overproduced in the *get2Δ sgt2-R171A, R175A* strain.

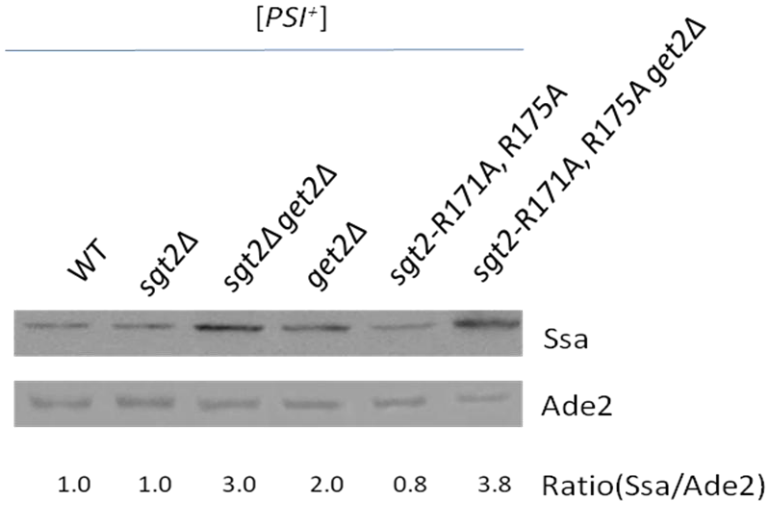


Figure 16 The effect of SGT2 deletion and mutation on Ssa1 levels
The levels of Ssa1 were compared in wild type and *get2*Δstrains, bearing *SGT2* deletion or mutation. Ade2 was used as a loading control. Result showed that *get2Δ* strains have excess Ssa1 comparing to those *GET2* strains.

Next we compared the efficiency of [*PSI*$^+$] curing in the same set of strains to examine the effect *sgt2-R171A, R175A* mutation. This experiment was performed in the qualitative curing of [*PSI*$^+$] by Hsp104 expressed under either P_{GPD} or P_{GAL} promoters. To overproduce Hsp104, those six strains were transformed with the plasmid pRS316/P_{GPD}-HSP104 containing *HSP104* gene under P_{GPD} promoter or plasmid

pGAL104-URA3 with *HSP104* gene under P_{GAL} promoter.

Four colonies were selected for each transformation and were patched onto media lacking uracil. For the first set, plates were incubated for ~ 3 days, and then were replica-plated onto YPD, media lacking uracil, media lacking adenine and uracil, and YPG. The curing was estimated by examining the growth on media lacking adenine and uracil after ~5 days. For the second set, plates were incubated for ~ 3 days, and then were velveteened onto YPGal media and incubated for one day. Cells were then repatached on media lacking uracil and incubated for 2 days. These plates were then velveteened onto YPGal again (2^{nd} round induction) and incubated for one day. Cells were then repatached on media lacking uracil. These plates were incubated for ~ 3 days, and were then replica-plated on YPD, media lacking uracil, and media lacking adenine and uracil. The curing was estimated by examining the growth on media lacking adenine and uracil after ~5 days.

In the both sets of the experiment, we observed the similar results (see Figire17). The comparison of the strain *sgt2-R171A, R175A* with wild type control shows that the disruption of interaction between Sgt2 and chaperones does not have its own effect on either maintaining [PSI^+] status or [PSI^+] curing by excess Hsp104. By comparing the

get2Δ sgt2-R171A, R175A strain with the *get2Δ*strain and the wild type control, it shows that this *get2Δ sgt2-R171A, R175A* strain also has a defect of [PSI^+] curing and suggests that the interaction between Sgt2 and Ssa is not needed for the protection of [PSI^+] from curing. Taking these results together with the Sgt2 and Ssa levels of these strains, a conclusion could be made that the protection of [PSI^+] from curing in *get2*Δ strain depends on the simultaneous overproduction of Sgt2 and Ssa. The direct interaction between Sgt2 and Ssa is not needed to observe this effect**.**

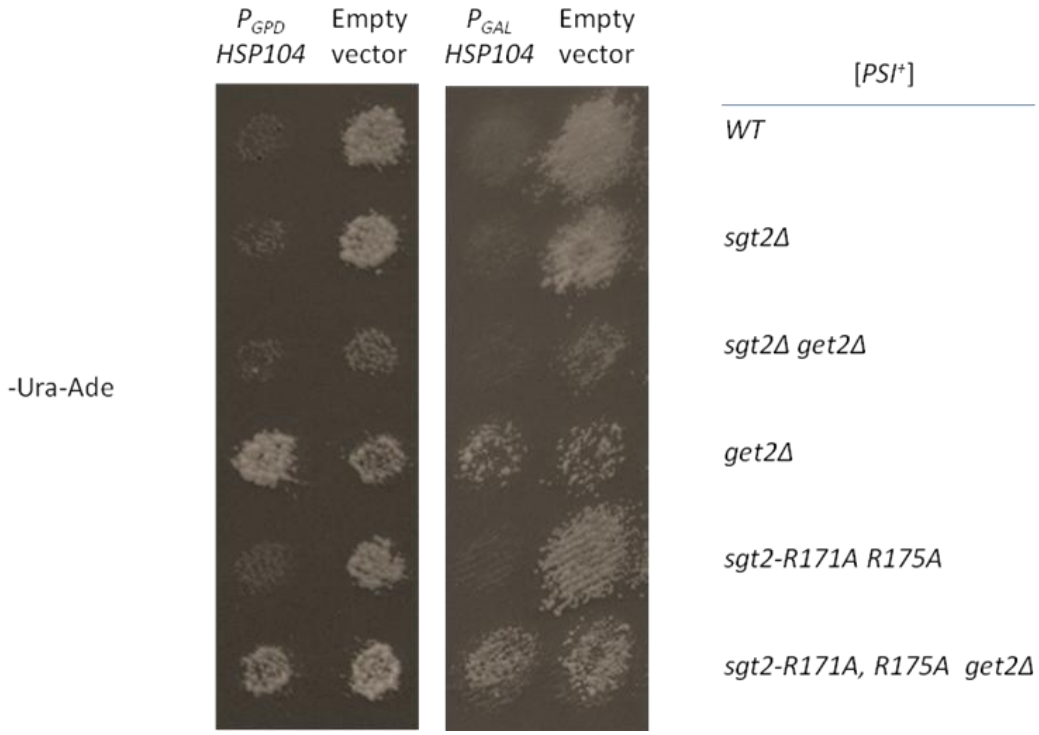


Figure 17 The effect of disrupting the interaction between Sgt2 and chaperones on [PSI$^+$] curing

Comparison of the efficiency of [*PSI*$^+$] curing in wild type and *get2*Δ strains lacking Sgt2 or having mutant form of Sgt2. Expression of *HSP104* was controlled by either strong constitutive (P_{GPD}) or strong inducible (P_{GAL}) promoters. In the first case strains were transformed with pRS316/P_{GPD}-HSP104 or control plasmid, patched on-Ura media and incubated for ~3 days. The -Ura plate was replica plated to -Ura-Ade and incubated for ~5 days. In the second case strains were transformed with pGAL104-URA3 or control plasmids, patched on -Ura media and incubated for ~3 days. The -Ura media was replicated to YPGal and incubated for 1day. Cells were then repatched on –Ura and incubated for 2 days. The -Ura media was replicated to YPGal again and incubated for 1day. Cells were then repatached again on –Ura and incubated for 2 days. The -Ura plate was replica plated to -Ura-Ade and incubated at 30℃ for ~5 days. The results show that by themselves, both *sgt2*Δ and *sgt2-R171A, R175A* have no effect on [*PSI*$^+$] curing. In *get2*Δ strains, *sgt2*Δ has a restoration to the defect of [*PSI*$^+$] curing but *sgt2-R171A, R175A* doesn't.

4 Discussion

Multiple works from different labs suggest that prion [*PSI*$^+$] is controlled by the ensemble of chaperones with Hsp104 playing the major role[42,50,51]. Changes in the chaperone balance affect [*PSI*$^+$] stability, and/or induction, and/or curing. The previous work performed in the Chernoff's lab showed that the defect in the GET pathway caused by *get2*Δ led to the defect in [*PSI*$^+$] curing by excess Hsp104. In this study we prescribe the mechanism underlying the effect of *get2*Δ on [*PSI*$^+$]. We demonstrate that overproduction of Ssa and Sgt2 is triggered by inactivation of the GET pathway and leads to the protection of [*PSI*$^+$] aggregates from curing. The Sgt2 overproduction is required in this case since a big part of Sgt2 may be sequestered by the TA/GET aggregates.

The initial step of this chain of events is a deletion of the *GET2* gene needed for the proper delivery of the TA proteins to the endoplasmic membrane. The exact mechanism that triggers the Ssa and Sgt2 induction is unknown. We believe it has a direct connection to the formation of the aggregates of TA proteins in the cytoplasm of the mutant cells. TA proteins have a stretch of hydrophobic amino acids on their C-terminal end[52]. Normally, these proteins are delivered rapidly to the ER membrane and are anchored into it. If GET pathway has a defect in function, the TA proteins are

expected to be accumulated in the cytoplasm[36]. Due to the presence of the exposed hydrophobic stretches TA proteins form aggregates that also include TA-interacting proteins. Sgt2 is a protein that normally interacts with the hydrophobic tail of TA proteins and it is sequestered in the GET/TA aggregates (Chernoff's lab unpublished data) together with Get3, Get4, and Get5. Ssa chaperones are able to bind hydrophobic sequences of the misfolded proteins and assist in their refolding or targeting to a certain compartment [30,53,54]. We believe that formation of the GET/TA aggregates triggers the non-specific cellular stress response and overproduction of the Ssa in order to protect the cell from accumulation of the misfolded proteins. This hypothesis is supported by the fact that Hsp104 is also overproduced in *get2*Δ cells (Chernoff's lab unpublished data). It is still not clear if Sgt2 overproduction is a result of the non-specific stress response in the cells or it is triggered specifically by the defect in the GET pathway. Either way, our data show that the direct interaction between Ssa and Sgt2 is not needed to protect [*PSI*$^+$] from curing. So, Sgt2 is not a co-chaperone of Ssa at least in Ssa interaction with [*PSI*$^+$] aggregates. To explain their combined action on [*PSI*$^+$] we propose the following model (Fig.18). We assume that Sgt2 protein changes the conformation of the β-sheet core of [*PSI*$^+$], which makes the Ssa protection from Hsp104 curing more efficient.

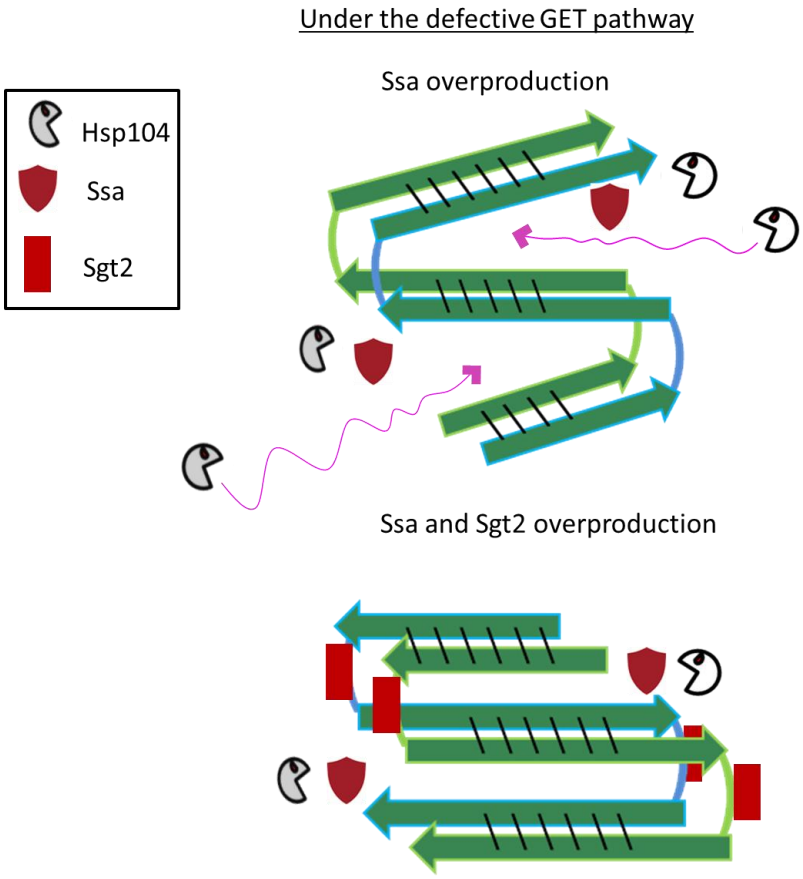


Figure 18 Model for Sgt2's role in the prion curing by Hsp104
Sgt2 protein changes the conformation of β-sheet of [*PSI*$^+$], which makes the Ssa protein easier to protect the fibrillar structure from Hsp104 curing.

This mechanism of [*PSI*$^+$] protection has a universal mechanism and can be observed not in *get2*Δ strains only. The deletions of components of the GET pathway, including *get1*Δ, *get3*Δ, *get4*Δ, and *get5*Δ, all exhibit the similar defect of [*PSI*$^+$] curing. We also showed that Sgt2 is overproduced to a different extent in all of these strains. Moreover, the Ssa levels are also higher in all of *get*Δ strains (Chernoff's lab unpublished data). Taken together, our data show a good correlation between the high levels of Ssa and Sgt2 in *get*Δ strains and the efficiency of [*PSI*$^+$] curing.

More experiments are to be done to examine our conclusion and model. First we need to find the domain of Sgt2 responsible for the interaction between Sup35 and Sgt2 and the positions of amino acids crucial for it. A strain with the disrupted interaction then will be created and we will check the [*PSI*$^{+}$] curing under Ssa overproduction. Afterwards, we need to create a strain with disrupted interaction between Sup35 with Sgt2 in *get2*Δ then check the [*PSI*$^{+}$] curing in this strain. If we are correct, strains with disrupted interaction between Sup35 with Sgt2 will have the same phenotype just like *sgt2*Δ stains. The deletion of Ssa will be not be adopted to this study since single deletion of any of the Ssa protein will not have any effect and a total deletion of Ssa will cause cell death. However, we can construct plasmid with mutated SSA having disrupted interaction with Sup35 or Hsp104 under strong constitutive or inducible promoter. For this purpose, we also need to find out the position of amino acid in *SSA* responsible for that. Overproducing these mutated Ssas and normal Sgt2 at the same time can help us clarify how Ssa protect [*PSI*$^{+}$] together with Sgt2.

Ssa may not be the only chaperone involved this Sgt2-chperones-[*PSI*$^{+}$] machinery dependant on defective GET pathway. Unpublished data form Chernoff's lab had already shown that Hsp104 maintains its function and Hsp40s are not involved in this

mechanism. But we haven't excluded the possibility that there are other chaperones involved in. It is known that the Ssb can facilitate the curing of [*PSI*$^{+}$]. Ssb overproduction increases, while Ssb depletion decreases, [*PSI*] curing by the overproduced Hsp104[42]. Although it is shown that no Ssb is observed in GET/TA aggregates (Chernoff's lab unpublished data), there is still a chance that Sgt2 can block the function of Ssb to halt the curing process.

In the Y2H experiment, the Sgt2 self interaction could be detected only in the haploid strain. This self interaction is not confirmed yet and this is one of the important issues to know how Sgt2 functions. If Sgt2 interacts with itself, the conformation change of the dimer of polymer form of Sgt2 may be responsible for changing the conformation of [*PSI*$^{+}$]. BiFC and FRET are potential methods to answer this question[55 56]. Moreover, constructions of plasmids with the full length *SUP35* as *Prey* and truncated *SGT2* as either Prey or Bait are important tasks in the future. Full-length Sup35 construction as Bait didn't provide us interpretable data, and we need to verify the interaction between Sgt2 and full-length Sup35. Constructions of truncated *SGT2* with only N or TPR or C domain will provide us information about in which domain Sgt2 interacts with Sup35.

However, Y2H could only be used to detect the interaction between Sgt2 and native

form of Sup35. Constructions of strains with truncated Sgt2 tagged with HA will provide us information of in which domain Sgt2 interacts with [*PSI*$^{+}$] by Co-IP method.

The results (see Chapter 3.2.2 and Figure 14) suggest that not only GET pathway affects [*PSI*$^{+}$] but also [*PSI*$^{+}$] may affect GET pathway. Since Sgt2 is induced in [*PSI*$^{+}$] strains, the function of GET pathway may be influenced by this effect.

5 Conclusions

I. Sgt2 interacts with Sup35 in both [*PSI*$^{+}$] and [*psi*$^{-}$] strains

II. Sgt2 interacts with Sup35N

III. Sgt2 levels are higher in the strains with the defective GET pathway

IV. Sgt2 assists Ssa in protection of [*PSI*$^{+}$] from curing

V. The direct interaction between Sgt2 and Ssa is not required for the protection of [*PSI*$^{+}$] from curing

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